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Sympathomimetic Activity in Extracts of Normal Human and Bovine Blood.

By

U. S. v. EULER and C. G. SCHMITERLÖW.

Received 26 August 1946.

The occurrence of pressor activity in blood and plasma, or in extracts thereof, has been frequently investigated, though so far hardly any consistent results can be collected from the vast literature on the subject. Usually, normal blood has been found to produce no action or to cause a lowering of the blood pressure.

Pressor actions of blood plasma have been reported by PAGE (1935), who also stated that the active principle was soluble in alcohol, water, acetone and chloroform. It did not appear in ultrafiltrates, but was liberated when the colloids were coagulated with alcohol. The active substance was fairly thermolabile. Similarly EULER and SJÖSTRAND (1944) found pressor activity in alcoholic extracts of human blood and plasma and in their dialysates. On account of the inconsistency of the pressor action on different test animals, and its contamination with depressor activity, it has not been possible to characterize the active agent with any greater degree of accuracy. A survey of the literature on the subject will be found in the last-mentioned paper.

Recently it has been shown that extracts of most mammalian organs contain considerable amounts of a sympathomimetic substance, differing from adrenaline but revealing close similarities with nor-adrenaline. This agent also occurs in large amounts in adrenergic nerves, and it is inferred that it constitutes the adrenergic neurohormone (sympathin) (EULER 1946 a, b, c.). For the demonstration of the characteristic actions of the sympathin, it

was found essential to remove contaminating depressor substances which was made possible by the aid of Fuller's earth.

By using this means of purifying the extracts biologically, it was hoped to obtain further information as to the nature of the pressor activity previously demonstrated in blood extracts.

Methods.

As material, human and bovine blood were used. Bovine blood was collected at the slaughter-house and heparinized. To the blood were added 2 volumes of alcohol and 5—7 ml 10 N sulphuric acid, per litre blood. After thorough mixing and standing for 2 hours, the blood was filtered and the filtrate concentrated in vacuo to a small volume. The reaction was adjusted, when necessary, to pH 4. Lipids were removed with ether, and the bulk of inorganic salts were precipitated by addition of 5 volumes of ethanol. After evaporation of the alcohol the aqueous solution was adjusted to correspond to about 100 ml blood per ml. The preparation thus obtained produced a considerable lowering of the blood pressure in the cat, and was therefore treated at pH 4 with $\frac{1}{5}$ volume of a 20 per cent suspension of Fuller's earth in water. Usually this treatment had to be repeated in order to remove the depressor activity.

Human blood was obtained from arm veins of medical students and Hospital blood donors and treated in the same way.

In some cases the lipid ether solution, obtained by extraction of the evaporated alcoholic blood extract, was shaken with a small volume of a 5 per cent sodium sulphate solution, which, after separation, was freed from inorganic salts by the addition of 3 volumes of alcohol. By this procedure a certain amount of pressor activity could be recovered, in conformity with the results previously reported for spleen extracts (EULER 1946 a).

Cats under chloralose anaesthesia were used for the blood pressure tests. The sensitivity of the animals was increased by injection of 0.1 mg Gynergen per kg and 8 mg cocaine hydrochloride per kg. For comparison with the pressor action of the extracts, adrenaline or dl-nor-adrenaline¹ was used.

In some cases, the isolated rabbit's intestine and the non-pregnant cat's uterus were used for the biological tests.

Results.

Bovine blood. In those test animals which reacted well, the extracts treated with Fuller's earth caused a rise in blood pressure

¹ Dr. H. Blaschko, of Oxford, kindly placed a sample of this substance at our disposal.

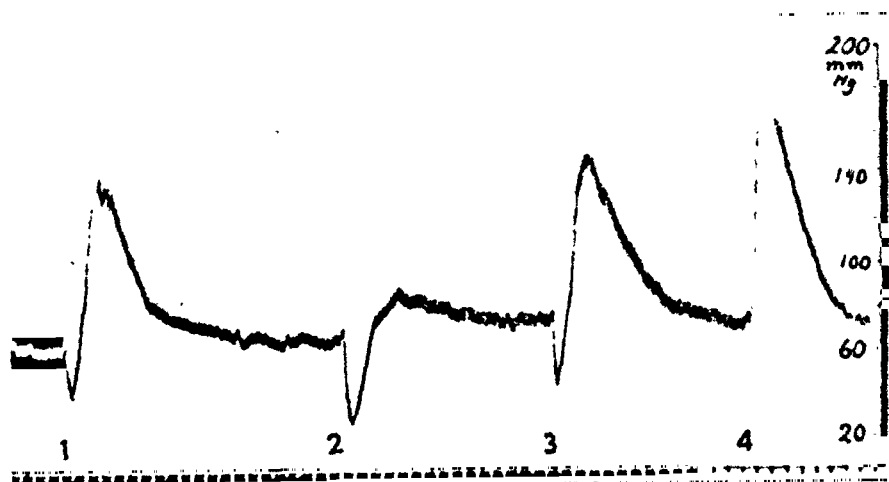


Fig. 1. Blood pressure, cat, chloralose, 0.1 mg/kg Gynergen, 6 mg/kg cocaine hydrochloride i.m., 20 mg Novocaine intramedullary.

1. 25 ml cattle blood extract, treated once with Fuller's earth.
2. same, treated with iodine at neutral reaction for 3 min.
3. same as 1.
4. 1 μ g dl-nor-adrenaline.

Time 30 sec.

accompanied in most cases by tachycardia (Fig. 1.). When compared with adrenaline and nor-adrenaline, a certain difference in the general type of pressor action could be noted in most cases. Thus the quick rise resembled that of nor-adrenaline and differed from adrenaline.

The quantitative estimation of the pressor equivalents of the extracts as expressed in adrenaline, or preferably, in nor-adrenaline, is rendered somewhat inaccurate by the Fuller's earth treatment which may have removed some of the pressor activity. The figures obtained are therefore probably somewhat too low.

The amount of pressor activity in bovine blood has corresponded to about 2—4 μ g nor-adrenaline per 100 ml blood when the purified extracts were used.

As to the active substance, it agreed with a sympathomimetic substance not only in producing an elevation of the blood pressure and in most cases tachycardia, but it was also enhanced in its action by cocaine to the same amount as nor-adrenaline. On addition of a slight excess of a n/10 iodine solution at neutral reaction, up to the point of a slight yellow colour, the activity disappeared. Pre-treatment of the animal with a sufficient dose of dihydro-ergotamine (ROTHLIN, 1944), decreased or abolished the pressor action of the blood extracts, as shown in the next section.

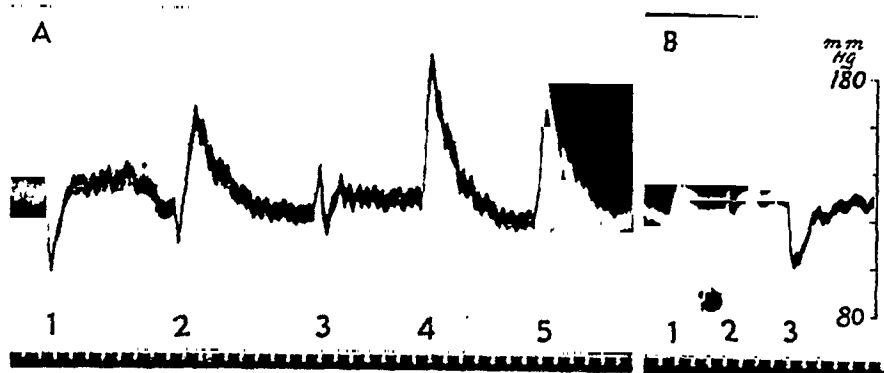


Fig. 2. Blood pressure, cat, chloralose. 0.1 mg Gynergen/kg, 8 mg cocaine hydrochloride/kg i.m.

- A. 1. Extract of 50 ml human blood, treated once with Fuller's earth and iodine at pH 8.
 2. 25 ml human blood extract as in 1.
 3. 0.5 μ g adrenaline
 4. 25 ml human blood extract, treated twice with Fuller's earth.
 5. 0.7 μ g dl-nor-adrenaline.
- B. (after 0.6 mg dihydro-ergotamine/kg).
 1. 2 μ g dl-nor-adrenaline
 2. 75 ml human blood, same extract as in A: 4.
 3. 2 μ g adrenaline.
 Time 30 sec.

Human blood. Normal human venous whole blood was extracted as described above. The extract after evaporation of the alcohol and removal of lipids was treated with 3 volumes of methanol and 3 volumes of ethanol, the precipitate washed with the same mixture, filtered, and freed from alcohol.

After adsorption twice with the Fuller's earth suspension, the extracts produced an almost pure pressor action of the same type as nor-adrenaline and typically differing from adrenaline. In one animal the response to a dose of 1 μ g adrenaline was a pure fall before cocaine, changing to a small double pressor reaction after cocaine, whereas nor-adrenaline and the blood extracts caused a simple quick rise in pressure (Fig. 2.). The pressor action was enhanced by cocaine, and destroyed by iodine at neutral or slightly alkaline solution. At pH 5 the inactivating ability of iodine was weaker.

Dihydro-ergotamine has been shown by ROTHLIN to be considerably more efficient in inhibiting the pressor action of adrenaline than ergotamine. A dose of 0.6—0.8 mg/kg, or even less, intravenously, generally caused a complete reversal of the adrenaline action on the cat under chloralose anaesthesia. As shown in

Fig. 2 a dose of 0.6 mg dihydro-ergotamine per kg. while reversing the adrenaline action, did not cause a reversal of the action of the blood extracts or nor-adrenaline, but reduced their action almost to annulment. This may be regarded as characteristic for the action of compounds of the nor-adrenaline type, and in view of the previous findings of a *nor-adrenaline-like action* in extracts of organs and adrenergic nerves (sympathin), it is highly probable that the action observed is due to sympathin and not to adrenaline.

The fluorescence test (GADDUM and SCHILD, 1934) was also employed. The considerable fluorescence in the extracts before addition of alkali made a decisive reading difficult, but since this fluorescence had a blue tint and became rather weaker on addition of alkali, and no typical green fluorescence occurred after alkali as was the case after addition of adrenaline to the extracts, we feel safe in stating that the active substance was not adrenaline. If the latter substance had been present in the extracts, its amount must have been insignificant.

In a few cases the purified extracts were also tested on the isolated rabbit's intestine and on the non-pregnant cat's uterus. In both, the inhibitory effect was much less than that of adrenaline in a dose which gave the same pressor response. On the other hand there was close agreement between the action of the Fuller-treated extracts and nor-adrenaline.

Discussion.

Some previous investigators (including EULER and SJÖSTRAND) have been able to demonstrate pressor activity in extracts of normal blood though great difficulties have been met in the assay and preparation of the active agent. This appears to have been partly due to the relatively strong depressor activity of the extracts, which has obscured the reactions. Furthermore, in the assay on other test objects, such as surviving organs, the actions have been mixed and the results uncertain. These difficulties seem to have been overcome by the methods introduced in connection with the preparation of sympathin (EULER 1946 b, c) and consisting in 1) increase of the sensitivity of the blood pressure test by a small dose of Gynergen and a moderate dose of cocaine, 2) removal of contaminating depressor and smooth muscle stimulating substances by treatment with Fuller's earth.

The demonstration of a sympathomimetic substance in extracts of normal bovine and human blood, corresponding to a pressor activity of some 2—4 μg nor-adrenaline per 100 ml blood, but clearly different from adrenaline raises the question as to whether previous findings could be reconciled with our results. Probably, for technical reasons, it has not been possible earlier to demonstrate convincingly a pure pressor activity in blood extracts, and it does not seem feasible to compare our results with those in which evidence for pressor activity in connection with depressor actions has been produced.

A number of papers, however, have dealt with the colorimetric determination of adrenaline in blood. Two rather sensitive, and apparently specific, methods have mainly been used, viz. the fluorescence method of GADDUM and SCHILD and the colorimetric method of SHAW (1938). Using the former method KALAJA (1942) concluded that normal human blood contains 6—12 μg adrenaline per 100 ml blood, and LEHMANN and MICHAELIS (1942) give an average figure of 186 μg adrenaline per 100 ml blood. Recently JØRGENSEN (1945) claims that normal human blood contains on an average 6.8 μg adrenaline per 100 ml blood. Against these reports stand those of GADDUM and SCHILD who found it impossible to give any values, on account of the disturbing fluorescence caused by the proteins of the serum, and of HUEBNER (1940) who normally only found traces. In a critical survey of the technical details of the method, PORAT (1946) points out the difficulties of determining adrenaline in small amounts in serum on account of the high blank values. By using a protein precipitation method, he was able to determine 25 μg per 100 ml, but he obtained no evidence for the presence of adrenaline in blood.

SHAW, using his own method, found in human blood 16—20 μg per 100 ml, but since the proportional increase due to alkali was only 1.2 times, it appears doubtful whether it is due to adrenaline. RAAB (1943) has given a mean figure of 15.6 μg per 100 ml blood in man which is obviously comparable with the results of SHAW. His denominator of the specific ratio was 1.06 *i. e.* still lower than that of SHAW. BLOOR and BULLEN (1941) however, in a careful study, found no detectable amounts of adrenaline in human blood with a modification of SHAW'S method, and conclude that normal human venous blood probably contains less than 0.1 μg adrenaline equivalents per 100 ml blood.

Our results have shown that the chief sympathomimetic pressor

agent in blood is not adrenaline but, probably, nor-adrenaline. Since this substance gives only $\frac{1}{16}$ of the colour of adrenaline weight for weight (SHAW), this would imply that the amount of nor-adrenaline, if determined solely by this method, should amount to some 250 μg per 100 ml which would give the same colour as 16 μg adrenaline per 100 ml. This figure is obviously out of the question. If our figure of 2—4 μg nor-adrenaline equivalents per 100 ml blood be accepted, this would give a colour reaction of only 0.12—0.24 μg adrenaline equivalents per 100 ml blood, though no increase in colour due to alkali would occur. This result, on the other hand, is not in disagreement with the results of BLOOR and BULLEN.

Summary.

Alcoholic extracts of whole blood from cattle and normal human subjects produce a rise of blood pressure and tachycardia in the cat, after adsorption of depressor constituents with Fuller's earth. The active substance is inactivated by iodine in neutral or slightly alkaline solutions.

The pressor action is enhanced by cocaine, and inhibited or abolished but not reversed by a dose of dihydro-ergotamine sufficient to reverse the action of an equipressor amount of adrenaline.

The evidence presented supports the assumption that the normally occurring pressor agent in bovine and human blood is chiefly nor-adrenaline (sympathin).

The amount of sympathin found in bovine and human whole blood corresponds to some 2—4 μg dl-nor-adrenaline hydrochloride per 100 ml blood.

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On the Occurrence of Vitamin K in Plants.

By

H. DAM, J. GLAVIND and E. K. GABRIELSEN.

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I. Introduction.

The occurrence of vitamin K in the higher plants has been studied especially by DAM and GLAVIND (1938 a) and DAM, GLAVIND and NIELSEN (1940).

From the former work it appears that parts of plants not normally forming chlorophyll contain only relatively little vitamin K. Without exception the vitamin content is highest in the green parts of plants. Furthermore, their work indicates that the formation of vitamin K, like that of chlorophyll, in the great majority of plant species depends upon the light, though not entirely to the same degree, as there is a very small quantity of the vitamin in etiolated, chlorophyll-free parts of plants.

The results of the latter work also suggest that there is some connection between the distribution of chlorophyll and vitamin K in plants. The vitamin is present in the plastides (chloroplasts) whereas in the cytoplasm and the sap of the cells it occurs only in very small quantities or possibly not at all. Moreover seedlings of conifers, which in contrast to most other green plants are able to form chlorophyll without light, contain fairly considerable quantities of the vitamin when they germinate in the dark. Comparison of the content of chlorophyll and vitamin K in conifer seedlings exposed to light and to darkness respectively, seems to indicate an approximate proportionality between the quantities of these two substances.

Chemically, there is a certain relationship between vitamin K isolated from green plants (K_1) and chlorophyll. Like the chlorophyll pigments, vitamin K_1 contains the radical phytyl of the unsaturated alcohol phytol. Thus it is not impossible that some of the factors governing the formation of chlorophyll may also influence the formation of vitamin K_1 . On the other hand, the chlorophyll pigments never occur alone in the chloroplasts of green leaves, but always together with yellow pigments, xanthophyll and carotin, in fairly regular proportionate quantities. Further, it is a known fact that in yellow spotted leaves of *Codiaeum variegatum* there is almost as much vitamin K in the all-yellow, chlorophyll-free areas of the leaves as in the green chlorophyllous areas (DAM and GLAVIND 1938 a). This being so, it is not impossible that the formation of the vitamin in some way or other is also dependent on factors controlling the formation of the yellow pigments.

In the present work we have attempted to delve deeper into the problem of the occurrence of vitamin K in plants, especially in order to examine whether there is a constant ratio between the quantities in which the vitamin and the various chloroplast pigments are formed.

II. Technique.

Immediately after sampling the plant material was desiccated at room temperature in vacuo over calcium chloride. It was then pulverized and stored in the dark until the determination of the vitamin and pigment contents could be made.

1. Determination of total chlorophyll.

About 0.05 g of the dry plant material was ground in a mortar with sand moistened with methanol and a little calcium carbonate. The pigments were extracted with methanol, the extract was filtered through a glass filter (Schott & Gen. 3G3) and made up to 50 ml. with methanol. The extinction in the clear filtrate was measured with a PULFRICH photometer, using the colour filter S 66.6/3.5, which separates a spectral region in which chlorophyll pigments have a powerful absorption while the carotinoid pigments allow the light to pass without hindrance. In the measurement we employed a layer of liquid 1 cm. in thickness.

The photometer was adjusted by means of a chlorophyll preparation ($a + b$) made from *Aesculus hippocastaneum* leaves (molecular quotient $\frac{a}{b} = 2.7$ after WILLSTÄTTER and STOLL 1913). The purity of the preparation was checked by elementary analysis. As the measured

extinction for the chlorophyll pigments is not proportionate to the concentration, we plotted an adjustment curve which showed the connection between extinction and concentration (0—2.5 mg/50 ml.).

Since the extinction coefficients are not the same for the two chlorophyll components in the spectral region employed, and the molecular quo-

tient $\frac{a}{b}$ varies in leaves of different plants, the measurements are subject to a systematic error that is difficult to avoid. According to WILLSTÄTTER and STOLL 1913 the quotient for green leaves of different plants varies between 2.1 and 3.4, according to SEYBOLD and EGGLE 1937 between 2.5 and 4.9. But even with these fluctuations the error in the measurements of the total chlorophyll content does not seem to be particularly great.

2. Determination of carotin and xanthophyll.

About 5 g. of the dried plant material was ground in a mortar with 5 g. of quartz sand and a little calcium carbonate and extracted in a glass filter (Schott and Gen. 11aG3) with 33 per cent. freshly distilled acetone. The filtrate was thrown away. Extraction was then continued, first with pure acetone until it ran off colourless, and thereafter once with 90 per cent. acetone and twice with a small quantity of ether. The pigment extract was evaporated in vacuo at about 40° and with abundant ether, transferred into a separatory funnel; any water present was separated by adding a little sodium chloride. The ether was dried with sodium sulfate which was then filtered off. The pigment solution was then concentrated in vacuo to a volume of about 25 ml., whereafter the chlorophyll pigments were saponified in a separatory funnel with about 20 ml. 4*n* methanolic potassium hydroxide in nitrogen atmosphere. Water was added after two hours. The aqueous phase with the green pigments was separated and shaken with ether a couple of times. Finally, the combined ether phases with the yellow pigments were washed with water until free of alkali, dried with sodium sulfate and filtered.

The separation of carotin and xanthophyll was performed in the following manner (WILLSTÄTTER and STOLL 1913): After evaporating the ether solution the pigments together with 100 ml. petroleum ether and exactly 85 ml. methanol were transferred into a separatory funnel and shaken; 15 ml. water was then added. The mixture was allowed to stand until the methanol was quite clear. The methanol phase with the xanthophyll was separated off and the petroleum ether mixed with 90 ml. methanol. 10 ml. water was then added and, after standing for a while, the methanol was again separated off. The process was repeated once or twice with 46 ml. methanol and 4 ml. water. If necessary the combined methanol phases were shaken once with petroleum ether which was again shaken with 90 per cent. methanol. The combined petroleum ether phases with the carotin were poured into a 200 ml. volumetric flask, and absolute ethanol added, whereby the liquid became completely clear. The flask was then filled up to the mark and the content used for colorimetric determination. The combined methanol phases with the xanthophyll were mixed with about 100 ml. ether to

which was added abundant water, whereby the pigment passed into the ether. The methanol phase was separated off and the ether phase poured into a measuring flask. After clearing with absolute ethanol the flask was filled up and the xanthophyll determined by colorimetry.

For the determination of carotin and xanthophyll in the PULFRICH-photometer we used 5 cm. microcuvettes; 96 per cent. ethanol was used for the dilution. The carotin was measured with filter S 45. With this filter the extinction coefficient $E_{1\%}^{1\text{cm}}$ measured with pure carotin in 96 per cent. ethanol is 2360. The xanthophyll was measured with filter S 47, with which the extinction coefficient $E_{1\%}^{1\text{cm}}$ for a solution of pure xanthophyll¹ in 96 per cent. ethanol is 2070.

3. Determination of vitamin K.

The determinations were made biologically by DAM and GLAVIND's curative method (D. & G. 1938 b). The determinations were all made with vacuum-dried, pulverized material with which the chickens were fed. The quantity of vitamin K is expressed in D. & G. units (DAM and GLAVIND 1938 b).

III. The Vitamin K Content in Leaves of Normal and Subnormal Green Color.

The investigation was made on leaf material from pairs of closely related plants mutually differing in their ability to form chlorophyll. The leaves came from four bushes: *Sambucus nigra* L. (green leaves), *Sambucus nigra aurea* SWEET (light green leaves), *Acer negundo* L. (green leaves) and *Acer negundo auratum* SPAETH (light green leaves). We also employed leaves of the herb *Atriplex hortense* L. (green leaves) and its chlorina variety (light green leaves). Before the leaves were picked the bushes had been growing for two years in a garden under uniformly good conditions; the herbs were also cultivated under uniform conditions.

The *Sambucus* leaves were picked on August 13th., the *Acer* and *Atriplex* leaves on September 9th 1940. The pigment and vitamin determinations were made during the same year. The results are presented in Table 1.

This table shows that the light green leaves of the varieties contain much less chlorophyll than the normally green leaves of the parent species. The xanthophyll and carotin values of the

¹ Kindly placed at our disposal by Professor P. Karrer, Chem. Inst. Univ. Zürich.

Table 1.

Pigment and vitamin content per gram dry substance of green and light green leaves.

Plant	Color of leaves	Chlorophyll (a + b) mg.	Xanthophyll mg.	Carotin mg.	Vitamin K D & G units.
<i>Sambucus nigra</i>	green	12.2	0.90	0.42	ca. 1000
<i>Samb. n. aurea</i>	light gr.	7.1	0.73	0.33	500—800
<i>Acer negundo</i>	green	15.5	0.61	0.26	400—600
<i>Acer n. auratum</i>	light gr.	3.6	0.35	0.19	400—700
<i>Atriplex hortense</i>	green	18.1	0.87	0.33	ca. 500
<i>Atriplex h. chlorina</i>	light gr.	4.6	0.55	0.26	400—600

leaves are also lower in the varieties, but on an average the deviations from the content in the leaves of the parent species are relatively much smaller than the corresponding deviations of chlorophyll.

The contents of chlorophyll and vitamin K in the various types of leaves do not exhibit proportionality. If we take the normally green leaves separately, the vitamin content is smallest in *Atriplex* leaves which have the highest chlorophyll content, and highest in *Sambucus* leaves in which the chlorophyll content is lowest. Comparison of the leaves of the varieties with those of the parent species indicates that only in the *Sambucus* bushes the difference in the content of vitamin K may be comparable to that of the chlorophyll. In the *Acer* and *Atriplex* leaves the quantity of vitamin K is fairly equal in the normally green and in the light green leaves, despite the fact that the latter contain only 23 to 25 per cent. of the chlorophyll found in the leaves of the parent species. The same absence of any regularity in the relation of pigment to vitamin quantity is also found on making a comparison between the xanthophyll or carotin content on the one hand and the vitamin content on the other.

IV. The Vitamin K Content of Plants with Artificially Induced Chlorosis.

In conjunction with these investigations, in which the pigment variations in the leaf material were governed genetically, we have examined the content of vitamin K in plants whose formation of pigment was inhibited by iron deficiency.

The plant employed in the experiments was *Zea mays* earl Crosby. Germination took place in moist hardwood sawdust. On July 10th the maize plants, which had developed two leaves, were transferred to one-litre water-culture jars containing iron deficient (40 plants) and complete nutrient solutions (10 plants). The complete nutrient solution contained per litre of distilled water: 1.5 g. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.25 g. KCl, 0.25 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.10 g. KH_2PO_4 , 0.08 g. ferric citrate and, as micronutrients, 0.6 mg. H_3BO_3 , 0.4 mg. $\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$, 0.05 mg. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.05 mg. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. In both solutions the hydrogen ion concentration was adjusted to $\text{pH} = 4.5$. Cultivation took place in a greenhouse under good light conditions.

On August 4th, the 3rd, 4th and 5th leaves of all plants were picked. The leaves of the iron-deficient plants were yellowish-white with a faint greenish tinge along the veins; those of the plants in the complete nutrient solution were of a vigorous green. Samples of the roots of the two batches of plants were also taken at the same time.

The results of the analyses are shown in Table 2.

It will be seen from the table that the leaves of the iron-deficient plants have a much lower pigment content than those of the normally developed plants. The most marked change is that of the chlorophyll; in the chlorotic leaves the chlorophyll content is only 6 per cent. of the content of the normal leaves, whereas xanthophyll amounts to 21 and carotin to 10 per cent. There is also a considerable difference in the vitamin K content, for the chlorotic leaves contain only about a fifth of the quantity found in the normal leaves. Thus the difference of the vitamin content in the two kinds of leaves is less than the difference of the chlorophyll content or that of the carotin content. Only with regard to the xanthophyll does there seem to be agreement between the quantities of pigment and vitamin; but, on the background of the results shown in Table 1 this agreement can only be regarded as accidental. Thus the conclusion to be drawn is that this investigation no more than the one described in the above section provides any definite evidence of a simple correlation between pigment and vitamin K synthesis in the plants.

The analyses of the roots show that vitamin K occurs in almost equal (but small) quantities in the roots of both normal and iron-deficient plants, so that iron does not seem to have any direct influence on the formation of this vitamin in the roots.

Table 2.

Pigment and vitamin content per gram dry substance in normal and chlorotic maize plants.

Parts of plants	Nutrient solution	Chlorophyll (a + b) mg.	Xanthophyll mg.	Carotin mg.	Vitamin K D & G units.
Leaves	+ iron	24.0	1.13	0.41	1400—1800
Leaves	— iron	1.4	0.24	0.04	about 300
Roots	+ iron	0.0	0.0	0.0	about 50
Roots	— iron	0.0	0.0	0.0	about 50

The lower vitamin quantity found in the leaves when the plants are deprived of iron may perhaps be ascribed solely to the circumstance that chlorotic leaves have a greatly reduced carbon dioxide assimilation and production of matter in the chloroplasts compared with normally green leaves.

V. The Vitamin K Content of Panached Leaves.

As mentioned in the introduction, DAM and GLAVIND (1938 a) found that the vitamin-K quantity in panached (locally chlorophyll-free) leaves of *Codiaeum variegatum* is almost the same in the yellow areas as in the green. For the purpose of completing that observation we have examined the distribution of the vitamin in panached leaves with areas that were almost white, and furthermore examined the pigment content in *Codiaeum* leaves from the same clone which provided material for the earlier studies. The panached leaves with almost white areas were taken from a variety of *Coleus hybridus*, the leaves of which had mar-

Table 3.

Pigment and vitamin K content per gram dry substance in panached leaves.

Leaf parts	Chlorophyll (a + b) mg.	Xanthophyll mg.	Carotin mg.	Vitamin K D & G units
<i>Coleus</i> , green marginal areas .	18.0	1.45	0.56	500
<i>Coleus</i> , white central areas ...	0	0.06	0.02	50—70
<i>Codiaeum</i> , green areas	6.8	1.09	0.25	300
<i>Codiaeum</i> , yellow areas	0	0.15	0.02	250

ginal zones with a normal chlorophyll content and central areas that were white with a faint yellowish tinge.

The results (Table 3) show that there is a remarkable difference between the distribution of vitamin K in the differently colored leaf areas of the two types of panached leaves. In the chlorophyll-free areas of the *Coleus* leaves, where the xanthophyll and carotin content is not particularly high, the vitamin content is much lower than in the green marginal zones and does not differ much from the level observed for completely pigment-free parts of plants (maize roots, Table 2). On the other hand, in the chlorophyll-free areas of the *Codiaeum* leaves, where the content of yellow pigment is higher than in the white areas of the *Coleus* leaves, there is abundant vitamin K. Nevertheless we can find no simple proportionality between the quantity of yellow pigments and the quantity of vitamin, neither when comparing the chlorophyll-free areas nor when considering the quantities in which the pigments and the vitamin occur in the yellow and the green parts of the *Codiaeum* leaves.

VI. The Vitamin K Content during Decomposition of the Chlorophyll.

As an appendix to the investigations in the synthesis of pigments and vitamin K in the plants, we made analyses of the vitamin content of leaves in which the chlorophyll pigments are in course of decomposition. They were made with leaves of *Sambucus canadensis aurea* COWELL, *Sambucus nigra* L. and *Aesculus hippocastaneum* L. The leaves at the various stages of wilting or yellowing were picked from the same bush or tree.

On the *Sambucus canadensis aurea* bush the leaves have more or less the same chlorophyll content in all the shoots immediately after unfolding. In the course of time the chlorophyll content increases somewhat in the lowest shoots of the bush, whereas in the leaves of the top shoots the chlorophyll passes through a process of decomposition, whereby the color changes from light green to yellow. This degradation is particularly rapid when the light is strong. As regards this bush the leaves at the various stages of chlorophyll break-down were picked simultaneously, the light green ones being taken from branches just below the top shoots. The *Sambucus nigra* and *Aesculus* leaves were picked at various

Table 4.

Pigment and vitamin K content per gram dry substance during the chlorophyll break-down.

Plant	Date	Chloro- phyll (a + b) mg.	Xantho- phyll mg.	Carotin mg.	Vitamin K D & G units
<i>Sambucus canadensis</i> aurea	light green leaves	6.0	0.57	0.31	800
<i>Sambucus canadensis</i> aurea	yellow leaves	0.7	0.60	0.06	500
<i>Sambucus nigra</i>	August 13th	12.2	0.90	0.42	about 1000
<i>Sambucus nigra</i>	Sept. 20th	6.5	0.79	0.27	500—800
<i>Aesculus hippo-</i> <i>castaneum</i>	Sept. 20th	12.7	1.16	0.44	about 700
<i>Aesculus hippo-</i> <i>castaneum</i>	October 9th	4.4	1.40	0.24	800—1200
<i>Aesculus hippo-</i> <i>castaneum</i>	October 17th	0.5	1.69	0.19	1300—1500

dates during the autumn. In the former species the chlorophyll is only partly broken down before the leaf falls from the bush; in the latter this break-down is almost complete before the leaf falls.

The results shown in Table 4 indicate that simultaneously with the break-down of chlorophyll there is a reduction of the carotin content in the leaves. The changes in the xanthophyll content are more varied; it hardly changes at all in *Sambucus canadensis aurea*, in *Sambucus nigra* there is a relatively slight reduction, and in the *Aesculus* leaves there is a marked increase. It is probable that part of this increase is due merely to an alteration of the basis of calculation owing to the fact that in *Aesculus* leaves more dry substance migrates into the tree during the yellowing processes than in other leaves. However, it is also possible that some of the carotin is oxidized to xanthophyll.

As regards vitamin K, the content in the first two plants in the table decreases somewhat, but the reduction is not in proportion to the changes in chlorophyll content. In the *Aesculus* leaves there is an increase in the vitamin quantity during the yellowing process. This increase may partly be due to the alteration of the basis of calculation as stated above. Thus, as the vitamin content in some plants decreases, whereas in others it increases, though without keeping pace with the alteration of the pigment content,

it may be stated that here again during the break-down processes there is no simple correlation between vitamin K and the various chloroplast pigments.

VII. Discussion.

The results of these investigations confirm the observations made previously, viz. that the vitamin K content in plants is highest in green parts (DAM and GLAVIND 1938 a).

On the other hand, there is no basis for the assumption that the formation of vitamin K in the plants is correlated with the formation of chlorophyll in such a manner that the two substances are synthetized in almost constant ratio. Investigations of normally green and light green leaves of closely related plants (Section III) as well as the comparison of the vitamin K and chlorophyll contents of normal and iron-deficient chlorotic plants (Section IV) show clearly that the variation in the vitamin content has no regular connexion with the variation in the chlorophyll content. Although chemically there is a certain relationship between vitamin K and the chlorophyll pigments, and although the occurrence of both substances is associated with one and the same element in the plant cells, i. e. the plastides (the chloroplasts), we must assume that the factors which influence the formation of vitamin K and chlorophyll, respectively, and determine the quantities in which these substances occur, are not all same. This conclusion is further supported by the fact that the break-down of the vitamin and that of the chlorophyll during the withering processes of the leaves in the autumn proceed quite independently.

Nor do these investigations confirm a supposition that there is interdependence between the formation of vitamin K and that of the yellow pigments, xanthophyll and carotin, which always occur together with chlorophyll in the chloroplasts. This supposition was supported by the fact that relatively abundant quantities of the vitamin are formed in those parts of panached *Codiaeum* leaves in which the plastides contain only xanthophyll and carotin (Section V). On the other hand, we have been unable to demonstrate a correlation between the quantities in which the vitamin and the two pigments occur in leaves of different plants. Furthermore, the vitamin content in the chlorophyll-free areas of *Coleus* leaves, where nevertheless there is some xanthophyll and

carotin, does not rise much above the level found in plant organs that are completely pigment-free (roots, Section V). It therefore seems unlikely that the building-up processes of the vitamin and the yellow pigments should be governed by the same factors. As to the relation between the formation of vitamin K and of carotin, we know that carrots which contain large quantities of carotin in the plastides of the roots, do not form more vitamin K than what can be observed in plant organs free of pigment (DAM and GLAVIND 1938 a). As xanthophyll represents an oxidative stage of carotin, this fact also argues that the synthesis of vitamin K proceeds independently of the formation of carotinoid in the plastides.

According to the investigations hitherto undertaken on the formation of vitamin K in the higher plants, the greatest quantities occur in the green parts of plants that are exposed to light. It seems likely that the conditions prevailing in the working chloroplasts, in which there is a lively formation of various intermediate products during the carbon dioxide assimilation, are particularly favourable to the synthesis of vitamin K.

Summary.

The content of vitamin K, chlorophyll, carotin and xanthophyll was determined in the following plant organs: 1) leaves of closely related plants with different synthetization of chlorophyll, 2) leaves and roots of plants with iron deficiency chlorosis, 3) leaf areas of panached leaves, and 4) leaves in which the pigments were in various phases of break-down. The results do not indicate that there is any simple correlation between the synthesis and break-down of vitamin K and the various leaf pigments.

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A Bronchoconstrictory Action of some Bile Acids.

By

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It is well known that bile acids play a part in choleresis and in digestion and absorption in the gut. Other effects of bile acids in the organism have also been described. In the past the cathartic action of bile has been empirically known and made use of; the experimental finding that bile acids given orally stimulate the peristaltic movements of the gut has offered an explanation of this effect (for references see EMMELIN 1941, DACSO et al. 1945). The bradycardia which sometimes accompanies jaundice has generally been supposed to be caused by bile acids accumulated in the blood. This hypothesis has given rise to a great number of experimental investigations concerning the action of bile acids on the heart; several different effects have been described but the results do not seem concordant (for references see WAKIM et al. 1939, 1940). Bile acids have also been observed to act on the uterus (HOFBAUER 1928). In this paper a bronchoconstrictory action of some bile acids will be described.

Methods.

The experiments were carried out on cats and guinea-pigs. 22 cats were used, 8 decerebrated and 14 under chloralose. Drugs were injected through a cannula in a femoral vein. In some of the experiments the blood pressure in the femoral artery was registered with a membrane manometer. The bronchial tone was registered according to KONZETT and RÜSSLER (1940). As contractions of the respiratory muscles may



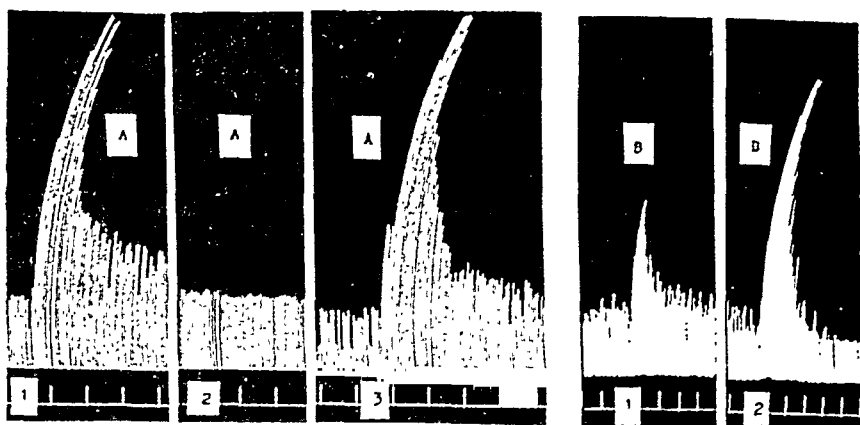


Fig. 2. A) Cat, 2.0 kg, chloralose. 1) 5 mg sodium glycocholate/kg injected in the femoral vein 2) the same dose in the portal vein 3) 50 mg glycocholate/kg in the portal vein. B) Cat, 3.2 kg, chloralose, 1) 5 mg glycocholate/kg in the femoral vein before and 2) after ligature on the hepatic artery and the portal vein. Time in minutes.

tion whereas 0.5 mg/kg is without effect. Taurocholate also elicits bronchoconstriction; 15—20 mg/kg has a marked effect, 1 mg/kg has none. Desoxycholate is active only in a highly toxic dose (about 50 mg/kg). Dehydrocholate even in an amount of 50 mg/kg has no bronchoconstrictory effect.

The conjugated bile acids are thus found to have the highest bronchoconstrictory activity. We have investigated the possible bronchoconstrictory action of glycine or taurine alone. In experiments where 5 mg of taurocholate/kg had a marked effect 10 mg of taurine had none; where 2 mg of glycocholate/kg caused bronchial constriction 10 mg of glycine/kg did not.

In guinea-pigs under urethane glyco- and taurocholate elicit bronchoconstriction. The sensitivity of the bronchi to these bile salts is found to be lower in these experiments than in the experiments on cats: 15—25 mg/kg of glycocholate and somewhat more taurocholate is required to obtain bronchial constriction.

Duration of the effect. The short duration of the bronchoconstriction caused by bile acids is striking. It is well known that bile acids injected intravenously are rapidly removed from the blood (for references see JOSEPHSON 1941). The part played by the liver can be well demonstrated in experiments of this kind. Fig. 2 shows that sodium glycocholate is about ten times less active on the bronchi when injected in the portal instead of the femoral vein. If on the other hand the liver is removed from the circula-

tion, the glycocholate, given in the femoral vein, elicits a much larger bronchoconstriction than it does when the liver circulation is intact. It is note-worthy that the duration of the effect is not markedly augmented in this experiment. This might indicate that other mechanisms play a part in the inactivation of bile acids. We have observed that a dose of glycocholate mixed with blood from the experimental animal a few minutes before the injection gives a smaller bronchoconstriction than the same dose dissolved in Tyrode solution.

Mode of action of bile acids. Several authors have maintained that bile acids stimulate parasympathetic centres in the medulla oblongata thus causing *e. g.* bradycardia in jaundice. The bronchoconstriction described in this paper, however, does not seem to be elicited in a similar way. The effect appears even if the vagus nerves are cut in the neck (fig. 1). HEBB (1940) has shown that in guinea-pigs the lung also receives bronchoconstrictory nerve fibres from the stellate ganglion. We have therefore in experiments on cats with sectioned vagi also removed the stellate ganglia and the 2—3 cranial thoracic ganglia on both sides. In these experiments bile acids cause bronchial constriction. (It might be added that we have observed only bronchodilatation, never constriction, after electrical stimulation of the stellate ganglion in the cat.) From our experiments it may thus be concluded that the bile acids act peripherally on the bronchi. This opinion is further supported by our observation that a certain dose of glycocholate elicits a greater bronchoconstriction when injected in the right ventricle than when given in the carotid artery. RIES and STILL (1933) claim that bile acids increase the sensitivity of certain effectors to stimulation of parasympathetic nerves. It is also established that bile acids can inactivate the acetylcholine esterase (SOBOTKA and ANTOPOL 1937). These findings, however, do not seem to explain the bronchoconstrictory action of bile acids. In cats with sectioned vagi we have injected glycocholate at a constant slow rate and in a concentration which causes only a slight bronchoconstriction; constriction elicited by electrical stimulation of the vagi is not found to be markedly increased by the glycocholate. Bile acids are also active in atropinized cat.

The bronchoconstrictory affect of bile acids is eliminated by injection of theophylline monoethanolamine (25 mg/kg); this drug is known to render the bronchi insensitive not only to vagal stimulation and acetylcholine but also to histamine (EMMELIN,

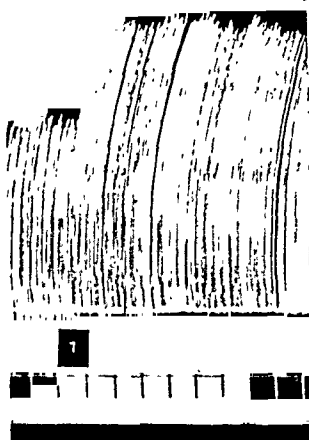


Fig. 3. Isolated guinea-pig's lungs, perfused with Tyrode solution. 1) Injection of 5 mg sodium glycocholate. Time in minutes.

KAHLSON and LINDSTRÖM 1941). Phenobarbitone also (50—100 mg/kg) antagonizes the bronchoconstrictory effect of bile acids.

The observation that bile acids elicit bronchoconstriction in isolated perfused lungs further supports the view that the point of action is situated within the lung (fig. 3).

Comment.

Some of the biological effects of bile acids seem to be related to the surface activity of these substances. ROTHLIN and SCHALCH (1944) comparing some bile acids found a close relation between surface activity, toxicity and hemolytic activity. With regard to these three properties desoxy- and taurocholic acids were found to be most active, glycocholic acid less and dehydrocholic acid least active. Choleresis and activation of lipase, on the other hand, are independent of these qualities. In our experiments the sequence is: glyco-, tauro-, desoxy-, dehydrocholic acid; this is true also when equimolar solutions are compared. Thus the bronchoconstrictory action of bile acids does not seem to depend on the surface activity, nor does there seem to be any relation to choleric or lipase activating properties.

Summary.

A constrictory action of some bile acids on cat's and guinea-pig's bronchi is described.

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Studies on Oxygen Treatment in Connection with Experimental Hydrocyanic Poisoning.

By

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According to the current pharmacological text-books the toxic effect of the cyanide ion is supposed to consist above all in a paralysis or blocking of the respiratory cell-ferments so that the oxygen absorption sinks (see e. g. GOODMAN and GILMAN). This leads to the arterialization of the venous blood, which was observed by CL. BERNARD and reported as early as 1857, and has since been analytically verified by many researchers.

Also cyanosis, however, is adduced as one of the typical symptoms of hydrocyanic poisoning, although this appears at a later stage of the poisoning. Assuming a normal hemoglobin value, this cyanosis must be due to a hypoxemia. And bearing in mind the generally injurious effect of hypoxemia, to which e. g. in modern shock research and anesthesiology much importance is ascribed, oxygen treatment should be valuable in connection with hydrocyanic poisoning (GORDH 1945). But besides this, oxygen treatment should be of particular importance in cases of hydrocyanic poisoning, for, as FERRALORO has shown, the toxicity of the hydrogen cyanide diminishes with rising partial pressure of oxygen, which may have some connection with the effect of oxygen on the cyanide poisoning of the carotid body (EULER, LILJESTRAND and ZOTTERMAN 1939). And finally, in an investigation carried out together with NORBERG, AGNER (1944) has found that in the course of hydrocyanic poisoning the oxygen

Table 1.

Animals that did not survive the acute poisoning with HCN in air.

Abbreviations: O₂S = oxygen-saturation in the blood;

O₂Res = artificial respiration with oxygen;

O₂ = oxygen.

The figures in parenthesis indicate the time in minutes from the commencement of the gassing.

Date	Rabbit no.	HCN mg/m ³	Gassing period in min.	Observations and treatment
20/7 1944	80	420	10½	The pulse began to become irregular after 9 min. Pulse scarcely palpable, O ₂ Res (12½). No effect.
24/11	94	558	6½	17 % O ₂ S (8). O ₂ Res (9). 30 % O ₂ S (15). Pulse not definitely palpable at conclusion of gassing.
	95	605	6	11 % O ₂ S (7). O ₂ Res (8). Dying pulse.
	101	577	5	5 % O ₂ S (6½). O ₂ Res (6¾). Dying pulse.
28/11	108	610	7	O ₂ Res, 8 % O ₂ S, only isolated heart-beats palpable (9).
	110	604	9	O ₂ Res (9½). 64 % O ₂ S (10). Only isolated heart-beats at beginning of treatment.
12/7 1945	121	284	30	21 % O ₂ S (33). O ₂ Res (34) but rapid worsening and death. The post-mortem showed pulmonary edema.

saturation in the blood sinks to values that are incompatible with the continuance of life, from which the conclusion has been drawn that the direct cause of death is the lack of oxygen, at least in cases of hydrocyanic poisoning through inhalation. The present investigation has been carried out in order to throw further light upon these questions.

Experimental Method.

The animals used for the experiments have been exclusively male rabbits weighing as a rule 2—3 kg. The gassing with hydrogen cyanide has been carried out with a gas mixture streaming at the rate of 30—100 l/min. in a small air-tight chamber with a capacity of 40 l. and provided with four holes with rubber cuffs, so that one or two persons could have their hands in the chamber during the gassing for purposes of control or operations on the animal. The concentration of hydrogen cyanide in the gas-mixture was always determined during the experiment by absorption of the nitril in sodab carbonate buffer according to KOLTHOFF and subsequent iodimetric titration. Gas pipettes of 0.6 l. were used for the taking of samples. For the oxygen treatment we

Table 2.

Animals that were gassed with hydrogen cyanide in air and in connection with which the oxygen treatment proved of value. Abbreviations as in table 1.

Date	Rabbit no.	HCN mg/m ³	Gassing period in min.	Observations and treatment
24/11 1944	96	521	3 ³ / ₆	49 % O ₂ S (4). O ₂ Res (4 ¹ / ₂). Breathes (5 ¹ / ₂). Reflexes + (6 ¹ / ₂). Head lifted, O ₂ cut off (9 ¹ / ₂).
	97	505	3 ³ / ₄	16 % O ₂ S (4 ¹ / ₂). O ₂ Res (5). Breathes (6). Reflexes + O ₂ cut off (8). Head lifted (13).
	98	553	5 ¹ / ₄	O ₂ Res, 44 % O ₂ S (6). 75 % O ₂ S (6 ¹ / ₂). Breathes (7 ¹ / ₂). Reflexes + (8 ¹ / ₂). Head lifted (18).
	102	635	5 ¹ / ₆	41 % O ₂ S (6). O ₂ Res (6 ¹ / ₂). 84 % O ₂ S (8). Breathes, reflexes + (13). Head lifted, O ₂ cut off (16). Lay dead in its hutch next morning.
	103	575	5 ¹ / ₂	23 % O ₂ S (6). O ₂ Res (6 ¹ / ₂). 85 % O ₂ S (9). Breathes, reflexes +, O ₂ cut off (10). Sudden death from heart tamponade (14) owing to lesion connected with the cardiac punctures.
9/7 1945	119	525	7 ¹ / ₂	Pulse weak. O ₂ Res (8 ¹ / ₂) immediately improves the pulse. Breathes (11). Reflexes + (17 ¹ / ₂). O ₂ cut off (20). Head lifted (30).

have used a plaster-of-Paris respiration mask modelled to fit the rabbit's muzzle and lined with chamois leather. In order to assure the passage of the gas through the rostrils narrow glass tubes have been inserted before fitting the mask. The respiration mask was directly connected with a rubber bag of 0.5 l. During the treatment the oxygen was supplied to the rubber bag at the rate of 3—5 l/min. With this arrangement it was possible to carry out the insufflation of oxygen by rhythmically squeezing the bag with the hand, i. e. artificial respiration with oxygen. When breathing spontaneously the animal obtained a high percent of oxygen through the mask.

In a large number of experiments blood tests were taken by cardiac puncture during the treatment for determination of the oxygen saturation according to JONXIS. In addition cyanide in the blood and rodanide in serum were determined according to a modification of WALLER's picrate method (NORBERG).

Results.

The course of the poisoning was very similar in the different experiments and developed as follows:

After one or two minutes the rabbit became uneasy. Imme-

Table 8.

Experiments with hydrogen cyanide in oxygen.

Abbreviations as in table 1.

A. Animals that died of acute poisoning.

Date	Rabbit no.	HCN mg/m ³	Gassing period in min.	Observations and treatment
21/11 1944	99	560	15 1/2	39 % O ₂ S (17 1/2), O ₂ Res (18), 56 % O ₂ S (19) dead (20).
23/11	112	521	24	O ₂ Res (24 1/2), 54 % O ₂ S, declining pulse (26). Despite apnea, only sporadic artificial respiration (28) 25 % O ₂ S (43) dead.
	114	555	15 1/4	45 % O ₂ S, 10 mg NaNO ₂ /kg, gasps (16), O ₂ (17), pulse does not recover (20), dead, 31 % O ₂ S (25).

B. Animals that survived the acute poisoning.

21/11	100	630	13 1/2	39 % O ₂ S, O ₂ Res (15), 58 % O ₂ S (16 1/4) breathes (22) O ₂ cut off (32), O ₂ supply resumed (35), reflexes + (39 1/4) stretching convulsions (45), O ₂ cut off (57), convulsions ceased (60). Was lively next day, but was found dead in its hutch on the morning of the third day.
	113	665	15 1/4	44 % O ₂ S, 1 mg Cu/kg (16 1/4), as pulse was not palpable, O ₂ Res started (17 1/2), breathes (20) reflexes + (26), oxygen cut off (30). Normal following day.

diately after this there were clonic and tonic spasms of varying intensity and frequently with opisthotonos, during which phase the blink and corneal reflexes as well as the reaction for a pinch in the ear ceased. The spasms were succeeded by apnea lasting for from one to several minutes, during which time the palpable cardiac activity — abbreviated in the protocol to "pulse" — was good. The apnea was in its turn generally succeeded by several minutes' sighing, slow and irregular diaphragmal respiration, "gasps". This was followed by fresh spasms and apnea. Fairly often nystagmus was observed and sometimes there was urination in connection with the spasms. Finally, the pulse became irregular and weak, at the same time as distinct cyanosis appeared (in the fundus oculi of albinos). Sometimes a sudden tachycardia was observed. In the control experiments these phenomena proved regularly to foretoken the definitive collapse. *The animals were thus kept in a hydrocyanic atmosphere until*

Table 4.

Experiments with hydrogen cyanide in oxygen. Abbreviations as in table 1.

Date	Rabbit no.	HCN mg/m ³	Gassing period in min.	Observations and treatment
22/7 1944	83	575	22	O ₂ Res (23), pulse immediately picked up. O ₂ Res stopped (26). Breathes (31). Owing to increasing cyanosis and weakening respiration, the supply of O ₂ was resumed (34). Reflexes + (53). Tonic extension cramps, oxygen cut off (55). After 2½ hours still slight cramps, but pulse and breathing good. Same status after 4½ hours. After 5 hours the rabbit was dead.
	85	450	34½	O ₂ Res (35½). Isolated breaths (36). Breathes O ₂ itself (43). Reflexes +, O ₂ cut off (64). After 2 hours unchanged status. After 3 hours the rabbit was dead.
	88	(1,200)	21	Itself breathes O ₂ (30). Reflexes +, O ₂ cut off (38). Oxygen supply resumed (43), cyanosis disappears. O ₂ cut off (80). Reacts to pinch in the ear (95). After 2½ hours reflexes extinguished again, and the rabbit died despite renewed oxygen supply.
	89	(920)	47	Itself breathes O ₂ (49). Reflexes + (54). Spasms (60). Spasms, O ₂ cut off (80). Reacts to pinch in the ear (100). After 2½ hours worse, and O ₂ supplied. Despite a spontaneous respiration of 60 gasps per minute, the rabbit could not manage without oxygen. When this came to an end after 4½ hours there was a rapid worsening and death.

complete cessation of respiration appeared, and the experiment was not discontinued until signs of circulatory collapse occurred with failing cardiac activity.

If, on the other hand, the supply of hydrogen cyanide was cut off at the cessation of respiration but while the cardiac activity was still good and there was only a moderate cyanosis, the rabbit recovered in fresh air without any measures being necessitated, even if the apnea had lasted rather a long time (even several minutes).

In order to test the value of the oxygen treatment, we have therefore endeavoured to carry the hydrocyanic poisoning to the point at which death was calculated to be imminent and inevitable. As a sign of an infaust prognosis we chose the failure of cardiac activity as described above, which according to our experience is both more reliable and sensitive as a criterion than the

Table 5.

The cyanide and rodanide values in blood after gassing with HCN in air.

Rabbit no.	HCN mg/m ³	Gassing period in min.	Time for test in min. from beginning of experiment	CN' in blood mmol	SCN' in plasma mmol
94	558	6½	8	0.058	0.095
95	605	6	7¼	0.050	0.102
96	521	3¾	4	0.064	0.142
			8	0.048	
97	505	3¾	4½	0.059	0.080
			7	0.054	
98	553	5¼	6	0.083	0.090
101	577	5	6¼	0.040	0.067
102	635	5¼	6	0.061	0.082
103	575	5½	6	0.036	0.107
			9	0.035	0.086
104	544	5½	6½	0.105	0.054
			12	0.107	0.056
			18	0.065	0.073
105	535	9	10¼	0.050	0.066
106	607	5	8	0.034	0.063
107	631	3¾	6	0.040	0.072
108	610	7	9	0.027	0.055
109	550	4	6	0.057	0.047
110	604	9	10	0.030	0.069
111	580	4½	7	0.023	0.051

respiration. It is, however, nevertheless difficult definitely to decide the right moment, so that in many cases the treatment was started when the circulation had already become too weak to enable the necessary transport of oxygen. Consequently, the percentage of successful treatments is low, to wit 50 % (18 experiments out of 36).

In tables 1 and 2 are given some typical experiments on oxygen treatment with and without good results.

The following points emerge from all the experiments:

The oxygen saturation in the blood is not in itself decisive for the result. The circulation must be sufficiently strong to convey fully saturated blood to the heart, which then, frequently already at the first insufflation of oxygen, begins to beat more strongly. In this case the oxygen saturation in the blood rises very rapidly (see experiments 98, 102 and 103 in table 2), while one otherwise gets a slow and slight increase (see experiment 94, table 1).

After artificial respiration with oxygen spontaneous breathing began, as a rule within one minute and on an average after 1½ minutes. The superficial reflexes returned 0—6 minutes, on an

Table 6.

Cyanide and rodanide values in the blood after gassing with HCN in oxygen.

Rabbit no.	HCN mg/m ³	Gassing period in min.	Time for test in min. from beginning of experiment	CN' in blood mmol	SCN' in plasma mmol
88	(1,290)	21 $\frac{1}{4}$	24	0.085	—
			155	0.033	—
89	(920)	47	49	0.108	—
99	560	15 $\frac{1}{2}$	17 $\frac{1}{2}$	0.080	0.078
100	630	13 $\frac{1}{2}$	15	0.077	0.118
112	521	24	26	0.056	0.060
			43	0.050	0.079
113	665	15 $\frac{1}{4}$	16 $\frac{1}{2}$	0.055	0.069
114	555	15 $\frac{1}{4}$	16	—	0.072
			25	—	0.093

average three minutes, after the resumption of spontaneous respiration. After a further 3—13, on an average 7 $\frac{1}{2}$ minutes the head was lifted.

In the hope of being able to throw further light on the question of the importance of oxygen gas in connection with hydrocyanic poisoning experiments were performed in which the animals were gassed with a mixture of hydrogen cyanide and *pure oxygen*. It was here possible to confirm FERRALORO's observations, as a fatal poisoning took twice as long to bring about as with HCN in air or even longer. In order to save the animal it was necessary, here too, to re-establish the oxygen saturation at a normal level. Some experiments illustrating this are given in table three.

Owing to the circumstance that the poisoning may go on for a longer time in oxygen, the injuries seem to get a irreversible character. For one thing, the period until spontaneous respiration and reflexes return is longer than after gassing in air, and for another thing the number of late deaths is greater. In table 4 are some further experiments to show this. In all four rabbits a very prolonged supply of oxygen was necessary before spontaneous respiration and reflexes returned. Normal motility, on the other hand, did not return; the animals lay on their sides and had from time to time slight spasms. After removal of the oxygen collapse occurred sooner or later and the animals soon died if the supply of oxygen was not resumed. This is especially striking in experiments 88 and 89.

The cyanide and rodanide analyses are given in tables 5 and 6

for the experiments. HCN-air and HCN-oxygen respectively. The dispersion is great in both the series, so that no importance can be ascribed to the, on an average, somewhat higher values for the oxygen animals. One can also see how the cyanide in vivo is slowly destroyed (rabbits 96 and 104 in table 5, rabbits 88 and 112 in table 6), but a corresponding increase of the rodanide-content is only indicated in a couple of experiments.

Discussion.

As hydrocyanic poisoning through inhalation is in its nature so acute and either proves fatal or is survived, as a rule without leaving a trace — according to the “all or none” principle — it is difficult for therapeutic attempts to find the narrow region between the self-healing phase of poisoning and the stage refractory to therapy. In spite of the subjectiveness of the decision of the point we consider that we have found very strong reason to assume that the supply of oxygen by insufflation may save the subject's life. The rapidity with which the effect appears puts all previously used methods of treatment in the shade.¹ And the simplicity of the oxygen treatment further enhances the value of the method.

By gassing in oxygen the tolerance is considerably increased. Despite the fact that considerably greater quantities of HCN are thus taken up in the body the content of HCN and SCN' respectively in the blood is not significantly increased. The oxygen obviously takes part in the detoxication of the hydrogen cyanide but not by furthering the formation of rodanide. Through the superintoxication in oxygen certain changes are made more acute and become irreversible. Thus, in several cases it occurred that several hours after recovery the animals were taken ill and died. This belated death could be postponed by supplying oxygen. These observations seem to support the assumption that the hydrogen cyanide in the first place inhibits the respiratory centre so that a deficient lung ventilation with hypoxia and finally cardiac insufficiency and death result. This gives further support to the theory that the oxygen treatment is rational.

¹ Comparative tests with nitrite and copper have been carried out and will be described in another connection.

Summary.

Rabbits have been poisoned by the inhalation of hydrogen cyanide in air or in oxygen. Signs of failing cardiac activity have been chosen as the indicator of the status ante mortem. Insufflation of pure oxygen in this stage saves the subject's life.

The experiments have confirmed the earlier assumption that the hydrogen cyanide poisons the respiratory centre, which leads secondarily to the anoxia that is the immediate cause of death. Oxygen treatment is therefore more rational and more rapid in its effect than the methods based upon detoxication of the hydrogen cyanide.

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On the Removal of the Lymphoid Organs and its Effect on the Blood Count.

By

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For the purpose of gaining some additional knowledge of the biological aspects of the lymphatic tissue we decided to try whether it would be possible technically to perform extensive extirpation of the organs belonging to this system in animals.

The difficulty of applying the classical method of extirpation in this field lies in the fact that the components of the system occur in large numbers and have a very scattered localization, one which formerly was even considered to be inconstant. What is more, it was a general assumption that the tissue had a well-marked power of regeneration which would rapidly compensate for any functional defect. This had never been proved, however, and recent investigations into the topography of the lymphoid organs have shown that there is constancy enough to enable one successfully to become trained in an operative technique for their removal.

In the course of our work we became acquainted with the successful attempts of SANDERS and FLOREY (1940) to remove very large amounts of the lymphoid tissue in rats and rabbits; from 87 to 93 per cent of the total organized lymphoid tissue as well as that in the spleen and thymus were removed.

We had realized in advance that a small part of the system which is difficult to evaluate must remain inaccessible to operative removal because of the diffuse distribution of its elements in

various mesenchymal tissues. But even with this limitation it was feasible to count upon inducing a manifest functional deficit by means of the proposed extirpations. It is well known from the reactions of other organs or whole systems that manifestations of deficiency appear long before the extirpations are total.

As a result of our operations we have primarily studied the number of blood lymphocytes as is affected by extensive reductions of the lymphatic system.

Operative Technique.

Guinea-pigs and rats were employed. As in other mammals, the lymphoid tissues occur partly in the form of separate lymphoid organs: lymph nodes, spleen and thymus, and also diffusely in some mesenchymal tissues, first and foremost in the mucosa of the alimentary tract and in the bone marrow.

Guinea-pigs. For the preliminary tests we employed guinea-pigs two to six months old, because in these animals thymectomy is easy to perform owing to the cervical position of the organ. As the work proceeded it became obvious that guinea-pigs were not particularly suitable; the laparotomies especially were difficult when they included the removal of mesenterial lymph nodes. Even when hemorrhage could be largely avoided the animals were almost always put into a state of shock. Subsequent peritonitis often set in.

Nevertheless it was possible to bring a few of the animals through by operating in two stages. At the first operation the thymus and the parotid lymph nodes were removed through an incision in the median line of the throat, and through incisions in the shoulder regions on both sides we removed the lymphonodi inframandibulares, submaxillares, praescapulares dorsales, cervicales profundae, retroscapulares and axillares (the nomenclature in conformity with HASHIBA, 1917). Two weeks later we removed through a median line incision in the abdominal wall lymphonodi abdomino-inguinales, hypogastricae, iliacae communes, aortae abdominales and mesentericae intestinales. By means of quite small incisions in the poples we were able to remove the popliteal lymph nodes. Working carefully and systematically we endeavoured to make the extirpations complete so that as far as possible no residue would be left in the regions where lymph nodes usually occur. One exception was two small nodes of the mesenterial group, these being left intact owing to the difficulty of getting access to them in the pancreas, nor did we venture to remove the nodes in the thorax: Lymphonodi inter-bronchiales and tracheo-bronchiales.

As a basis narcotic for anaesthesia we employed subcutaneous injection of sodium phenylethylbarbiturate in doses of 6 mg. per 100 g. body weight, with the addition of ether inhalation, especially when making the skin incisions. For the purpose of stimulating the animals,

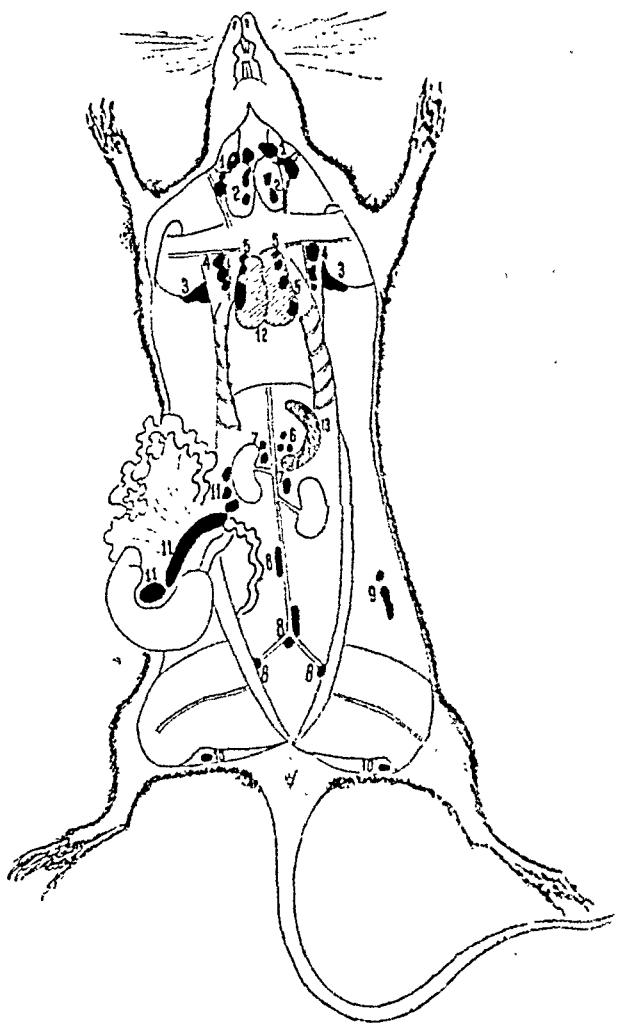


Fig. 1. The lymphoid organs of the rat.

- | | | |
|--------------------------|----------------|-----------------|
| 1. Superficial cervical. | 6. Cisternal. | 10. Popliteal. |
| 2. Deep cervical. | 7. Renal. | 11. Mesenteric. |
| 3. Brachial. | 8. Lumbar. | 12. Thymus. |
| 4. Axillary. | 9. Epigastric. | 13. Spleen. |
| 5. Thoracic. | | |

which were in a very poor state after the operations, injections of pentasol were given regularly.

Rats. For the actual experiments we employed white laboratory rats, as these tolerated the abdominal operations better. Another reason was that by doing so we could work with a material already specially examined as regards the normal anatomy of the thymo-lymphatic system (ANDREASEN 1943).

The operations were made in one seance. The superficial cervical lymph nodes were removed through an incision in the median line of

the anterior surface of the neck. After prolonging the incision down over the front of the thorax the manubrium sterni was divided with scissors exactly in the median line, whereafter the thymus was removed with forceps. Simultaneously bilateral pneumothorax occurs. By quickly compressing the resilient wall of the thorax and only allowing it to expand again after suturing, the collapsed lungs expand again sufficiently for the resumption of the respiration. In a few cases we removed the bronchial lymph nodes under positive pressure anaesthesia.

The brachial, axillary and deep cervical lymph nodes were next removed from the dorsal side through an oblique incision in both sides. Finally, through an incision in the median line we removed the mesenteric, lumbar and epigastric nodes, and lastly the spleen. It was possible to remove the popliteal lymph nodes through incisions in the poples.

The ether used for anaesthesia was administered exclusively through a special aggregate, whereby there was constant control of the proportions of ether and atmospheric air. The entire operation lasted about an hour. The animals were not exhausted after the operation, medicinal stimulation being quite unnecessary. After losing weight for a day or so the animals began to grow again and continued to do so normally, and in every other respect they were normal to direct observation.

We found it unnecessary to undertake any preliminary staining of the lymph nodes, as suggested by SANDERS and FLOREY. The rat strain available carried no latent infection with *Bartonella muris*, for which reason prophylactic treatment with neo-salvarsan against *Bartonella* anaemia after splenectomy was unnecessary.

The Effect of Extensive Lymphadenectomy on the Blood Count.

At various times before and after the operation the total number of white blood corpuscles was determined along with a differentiation between granulocytes, small lymphocytes and large lymphocytes. In the latter group we placed all large mononuclear cells without otherwise distinguishing between large lymphocytes and cell forms which perhaps should be regarded as monocytes. The blood examination also included haemoglobin determinations and erythrocyte counts. All determinations were made on blood from a tail vein, the animal being placed in the incubator at 40° for ten minutes to ensure good bleeding and uniform conditions with regard to the blood flow in the tail.

The blood picture was followed systematically in four subtotally operated animals, and, as a control on the specificity of

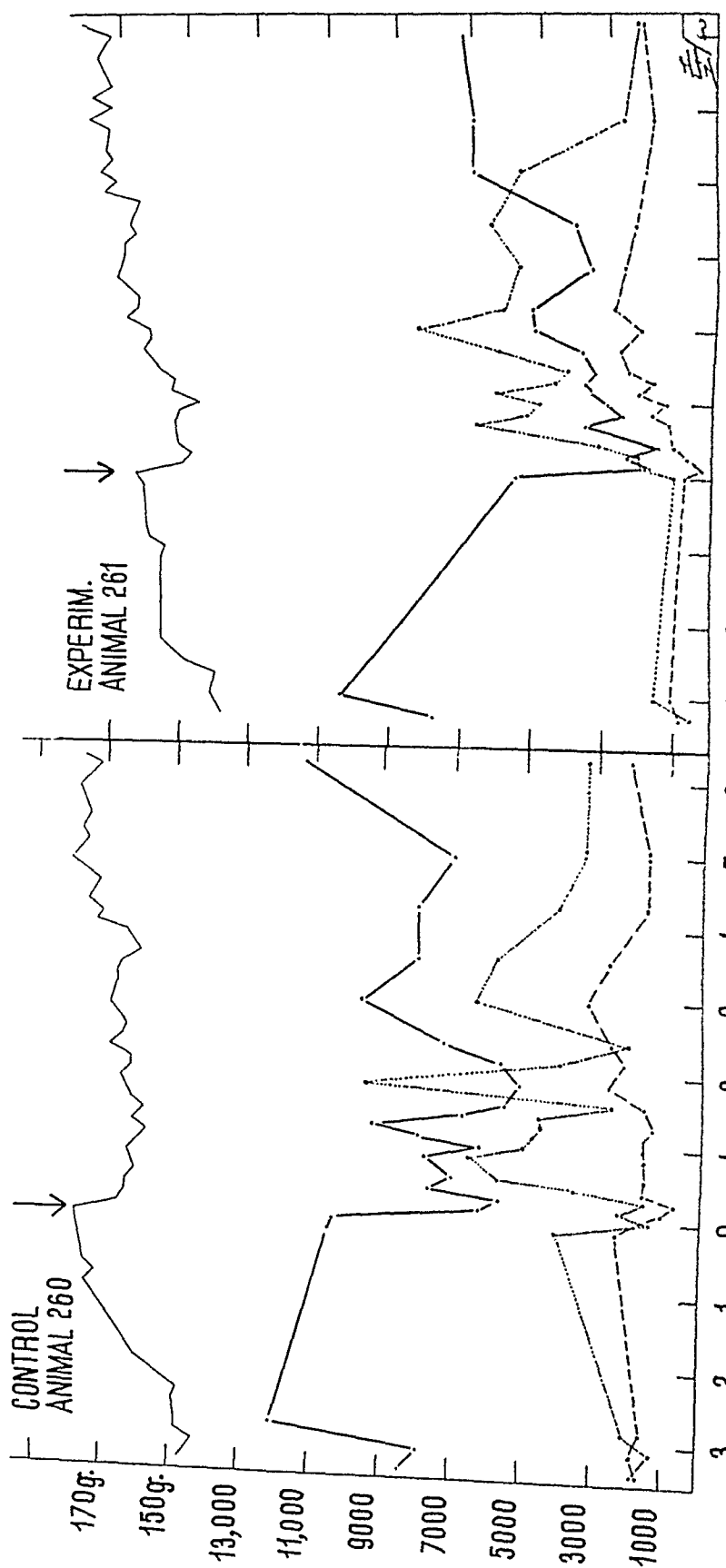


Fig. 2. Graphical presentation of the numbers of white blood cells after extensive lymphadenectomy (animal 261) and after sham-operation (animal 260).
 — small lymphocytes, - - - - - large lymphocytes, granuloocytes. The arrow indicates the day of the operation.

the effects, on four sham-operated animals, all females about 14 weeks old at the beginning of the experiment. As far as possible the control animals were subjected to the same conditions as the experimental animals with regard to anaesthesia, incisions, suturing and other treatment. The weight curves for the two groups also run fairly parallel.

The blood picture was observed for some time prior to the operations and for about six weeks after; in fig. 2 some typical curves are recorded. Even under quite normal conditions the lymphocyte counts vary a good deal in the same animal in conformity with earlier observations (ANDREASEN 1945). One conspicuous consequence of the operation is a marked lymphopenia occurring during the course of the first 24 hours. From preoperative values between 5,520 and 13,280 the drop went right down to between 360 and 1,800. On the very next day there was a slight increase to between 2,360 and 3,640. During the next three to six weeks the lymphocyte counts were still low, and the values only return to about the initial figures after that time.

There are no definite variations in the granulocyte counts during the days just after the operation, but on the fourth or fifth day a marked leucocytosis sets in, with values between 6,700 and 13,200. The course fluctuates somewhat, and the leucocytosis persists until three or four weeks after the operation.

After the control operations we usually found that after the first 24 hours there was some lowering of the lymphocyte count from an initial value between 4,080 and 12,000 down to between 3,280 and 6,240. This fall was quite transitory, however, and afterwards no definite deviation from the normal could be observed. Granulocytosis sets in moderately after a few days, but subsides in the course of a week or two.

Subtotal extirpation causes moderate anaemia with haemoglobin values down to 60 per cent and an erythrocyte count down to 4.3 millions. In the control animals too there was post-operative anaemia with values down to 70 per cent and 5.1 millions respectively.

In order to make closer observations of the lymphopenia occurring in association with the operation we made counts at short intervals within the first 24 hours on one of the subtotally operated animals and one of the controls. The results are given in fig. 3. The lymphocyte fall begins in the period immediately after extirpation, and the lowest value is reached about 7 hours

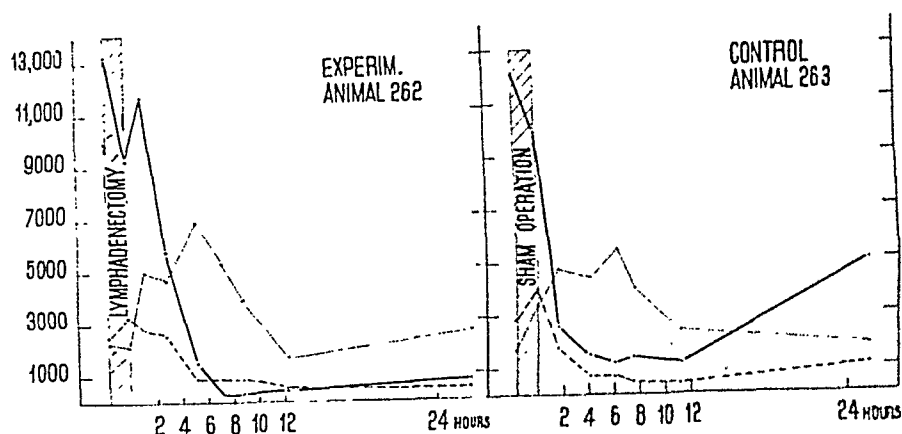


Fig. 3. Graphical presentation of the numbers of white blood cells after extensive lymphadenectomy (animal 262) and after sham-operation (animal 263).

— small lymphocytes, - - - - - large lymphocytes,
 granulocytes.

after the end of the operation, viz. 300 small lymphocytes per c.mm. blood. The values were almost unchanged during the subsequent hours, and 27 hours after the completion of the operation the count gave 800 lymphocytes per c.mm. The control operation was also found to be followed by a pronounced fall of the lymphocyte values, with the lowest, 1,200, at the same time as the subtotally operated animal. The value remained low during the following hours, to be followed by an increase to 4,980 after 26 hours. Granulocytosis of parallel course and lasting about 12 hours was observed in both animals.

Discussion.

If we try to summarize these observations the conclusion will be is that sub-total extirpation as performed by us induces a pronounced lymphopenia of short duration, with its lowest values in the first 24 hours after the operation; restoration to normal values occurs in the course of three to six weeks.

The specificity of the changes is elucidated by comparing with the course after the sham-operation; the lymphocyte falls in the first hours correspond so closely that we are constrained to assume that they are caused by unspecific factors and associated with one or more of the multiform influences to which the animals are subjected

Comparison of weights of removed lymphoid organs and those found post-mortem.

Animal		Thymus		Spleen		Lymph nodes		
No.	Body-weight	Extir-pated	At Au-topsy	Extir-pated	At Au-topsy	Extir-pated	At Au-topsy	Total
S u b t o t a l o p e r a t e d								
	g.	g.		g.		g.	g.	g.
256	160	0.206		0.432		0.335	0.121	0.456
258	155	0.160		0.317		0.210	0.069	0.279
261	164	0.178		0.309		0.293	0.080	0.373
262	179	0.217		0.345		0.351	0.091	0.442
Av.	165	0.190		0.351		0.297	0.090	0.387
S h a m - o p e r a t e d								
257	165		0.124		0.313		0.297	
259	162		0.138		0.350		0.354	
260	178		0.131		0.336		0.424	
263	172		0.180		0.393		0.504	
Av.	169		0.143		0.348		0.395	

during an operation. It seems that the organism is capable of eliminating this unspecific factor or factors in the course of the first few days. The most natural assumption would be that the effect is the result of a transitory inhibition of lymphopoiesis, as the lymphoid system is known to be extremely sensitive to interferences of the most varied kinds. There are several other possibilities, however, for instance increased emigration from the blood-stream or retention in the lymphoid tissues.

On the other hand, the more protracted lymphopenia found in the sub-totally operated animals may be regarded as being in causal relation to the reduction of the lymphoid tissue. Some measure of this is obtainable by weighing the extirpated organs (see table).

Normal values from a larger material examined previously (ANDREASEN 1943) show that the bronchial and popliteal lymph nodes left in place represent 6.65 and 1.55 per cent, in all 8.2 per cent of the total amount of lymph nodes. When this is put in relation to the total quantity of lymphoid organs, including the Peyer's patches, it means that 9.3 per cent is left behind (still

disregarding the diffuse lymphoid tissues). This weight determination, however, provides no adequate expression of the reduction in the lymphopoietic activity. Our knowledge of the nucleic acid conversion in the various lymphoid organs (ANDREASEN and OTTESEN 1945) shows that the lymph nodes representing these 9.3 per cent, are a less active part of the system.

Thus the degree of the lymphopenia is in no direct proportion to the very considerable reduction of the lymphoid system; the unknown quantity of the diffuse lymphoid tissues and the probably strongly fluctuating activity in the various components of the system make it impossible to arrive at a closer quantitative evaluation of this relation. Furthermore, the mechanism regulating the number of the blood lymphocytes is so obscure that one could hardly expect to find any direct parallelism.

The re-establishment of the normal blood lymphocyte values observed after three to six weeks after the extirpations is equally difficult to account for.

The question of the re-establishment of organoid lymphoid tissue was examined by means of weighing in conjunction with autopsy after a post-operative interval of about six weeks. We found no remnant of thymus or spleen, and the total quantity of lymph nodes averaged a weight of 0.090 g. (see table). In the control animals the total lymph nodes averaged 0.395 g. On comparing this with the sum of the extirpated nodes and the remnants found post mortem: an average total of 0.387 g., we find the values in close agreement. The obvious conclusion to be drawn therefore is that in the period of observation only slight quantitative changes, or none at all, took place in the total mass of the remnants, and certainly nothing like a re-establishment of the lymphoid tissue mass.

The granulocytosis observed seems mainly to be unspecific in nature and is probably caused by processes of wound healing, with which they coincident in time.

The post-operative anaemia disappeared spontaneously, at any rate within three weeks. This point could not be gone into more closely on these animals, as the constant blood sampling for the counting of white blood corpuscles is not without influence on the re-establishment of the normal haemoglobin percentage and erythrocyte value.

Summary.

The authors have endeavoured to adapt the classical method of extirpation, for investigating the function of a tissue, to the lymphoid system. Operative technique for the removal of lymphoid organs is described for use on guinea-pigs and rats.

In rats we found a pronounced lymphopenia lasting for three to six weeks in association with the operations. In the first days it was found to be unspecific, whereas during the remainder of the period it stands presumably in causal relation to the extirpation.

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Free Amino Acids in Dialyzed Casein Digest.

By

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In recent years, amino acids obtained by enzymic hydrolysis of proteins, especially of casein, have been used to an increasing extent both in medicine and surgery. ELMAN and WEINER (1939), HILL (1941), and SHOHL (1943) have used amino acid mixtures produced by digestion of casein by pancreatic enzymes. Protein is not to be found in these casein digests by chemical methods (SHOHL, BUTLER, BLACKFAN and MACLACHLAN 1939).

The amino mixture most frequently used in these American investigations is Amigen; this is an enzymic casein digest about 60 per cent of which, according to HILL (l. c.), consist of free amino acids. No information is available, however, as to which of the amino acids are free in this preparation.

WRETLIND (1944, 1945), MAGNUSSON (1944), LIDSTRÖM (1944), and JORPES, MAGNUSSON and WRETLIND (1946) used for their investigations an amino acid mixture prepared by a method worked out by WRETLIND (l. c.), which is based on digestion of casein by trypsin and erepsin followed by dialysis, which separates the amino acids and the peptides of low molecular weight from the proteins and the peptides of high molecular weight. This preparation¹ has been found, from the biological point of view, to be an adequate food able to cover the nitrogen needs of the body. When administered perorally to rats it gives both nitrogen equilibrium and a considerable degree of nitrogen retention

¹ Supplied under the trade name of Aminosol and prepared by Vitrum A.B., Stockholm. This preparation was used for the present investigation.

(WRETLIND 1946). LIDSTRÖM (l. c.) has demonstrated that nitrogen equilibrium occurs in man when this dialyzed protein digest (Aminosol) is given intravenously. In view of the possibility of obtaining nitrogen equilibrium by parenteral alimentation with a protein digest of this type, it seems of interest to ascertain to what extent the amino acids, and also which of them, occur in a free form. The casein digest is prepared from pure casein and the amount of purified enzymes added is about 2—3 per cent (reckoned as pure protein) of the amount of casein. In this casein digest the amino acids occur partly in the form of sodium salts, an aqueous solution of the preparation thus having a pH of 7.1—7.3.

Experimental.

Amount of Free Amino Acids in the Dialyzed Casein Digest.

In order to gain an idea of how far the splitting of the casein had proceeded, the amino nitrogen and the total nitrogen in the amino acid preparations before and after hydrolysis with 20 per cent hydrochloric acid for 24 hours were first compared. The percentage of peptides is shown, as proposed by HENRIQUES and GJALDBAECK (1911), as the percentual difference in the amino nitrogen before and after the hydrolysis with hydrochloric acid.

The amount of amino nitrogen was determined by VAN SLYKE's (1913) method, after removal of the ammonia by calcium hydroxide, the amount of ammonia by the method of VAN SLYKE and CULLEN (1914), and the total nitrogen by the micro-KJELDAHL method (PREGL 1930).

In the analyses of the dialyzed casein digest, preparations intended for peroral administration as well as preparations in solution for intravenous injection were used.

I. Casein Digest (Aminosol) for Peroral Administration.

A. Before hydrolysis with hydrochloric acid: 5,000 g of casein digest (Aminosol for peroral administration, not water-free) were dissolved and made up to 100.0 ml. with distilled water. Of this solution, 5.00 ml. were used for the determination of the ammonia, 5.0 g of potassium carbonate being taken for this purpose. — The solution containing the casein digest was diluted exactly 1 + 2 and from this 0.50 ml. was taken for the determination of the total nitrogen (micro-KJELDAHL). — To 10.00 ml. of the diluted solution 0.6 g of CaO was added, and the solution was then evaporated to dryness on a steam bath. The residue was dissolved in 1 ml. of glacial acetic acid and distilled water was

added to make 10.00 ml. — 1.0 ml. of this was used for the amino nitrogen determination.

B. *After hydrolysis with hydrochloric acid:* 5,000 g of casein digest (not water-free) was dissolved in 50 ml. of 20 per cent hydrochloric acid and boiled for 24 hours on a sand bath with a reflux cooler. It was then concentrated in vacuo and dissolved in distilled water to make 100.0 ml. after neutralization with 1 N sodium hydroxide. 5.00 ml. of this solution were used for determining the ammonia. After diluting exactly 1 + 2, 0.50 ml. was used for the KJELDAHL determination and for determining the amino nitrogen 10.00 ml. were treated in the same manner as the casein digest not hydrolyzed with hydrochloric acid.

II. Casein Digest (Aminosol) for Intravenous Injection.

A. *Before hydrolysis with hydrochloric acid:* The commercial 3.3 per cent solution was used for these determinations. 5.00 ml. were measured off for the determining of the ammonia, 5.0 g of potassium carbonate being used for this purpose. 2.0 ml. were used in determining the total nitrogen, which was done by a macro-KJELDAHL method (PETERS and VAN SLYKE 1932). To 10.00 ml. of the solution was added 1.2 g of CaO, and the solution was then concentrated to a small volume over a steam bath. The residue was dissolved and made up to 20.00 ml. with 2 ml. of glacial acetic acid and distilled water. From this solution 1.00 ml. was drawn off for determining the amino nitrogen.

B. *After hydrolysis with hydrochloric acid:* 100.0 ml. of the 3.3 per cent solution were concentrated in vacuo to a small volume, after which 30 ml. of 20 per cent hydrochloric acid were added, and this mixture was then boiled for 24 hours on a sand bath with reflux cooler. The hydrolysate was concentrated in vacuo and dissolved to make 100.0 ml., neutralization being obtained with 1 N sodium hydroxide. The total nitrogen, ammonia, and amino nitrogen were determined in the manner described under section A.

The results of these determinations are shown in table I. All the values have been converted into 1 ml. of undiluted (5 per cent or 3.3 per cent) solution.

It will be seen from table I that, both in the peroral and the intravenous casein digest, the relation between the amino nitrogen and the total nitrogen increases during acid hydrolysis from 0.54 to 0.71, and from 0.50 to 0.75, respectively, which implies the setting free of 24 and 33 per cent of amino acid groups. This means that in the casein digest (Aminosol) 76 per cent of the peroral preparation and 67 per cent of the intravenous one is composed of free amino acids. The ammonia nitrogen content in the peroral casein digest (2.4 % of the total nitrogen) is smaller than in the intravenous one (4.4 %). In the casein digest hydro-

lyzed with hydrochloric acid the ammonia nitrogen is only somewhat smaller in the peroral preparation (7.2 % of the total nitrogen) than in the intravenous one (7.5 %).

Table 1.

Preparation	Total nitrogen mg	Amino nitrogen mg	Ammonia nitrogen mg	Relation: $\frac{\text{amino nitrogen}}{\text{total nitrogen}}$
Aminosol for peroral use without acid hydrolysis..	6.02	3.25	0.146	0.54
Aminosol, for peroral use, with acid hydrolysis	5.88	4.17	0.423	0.71
Aminosol, for intravenous injection, without acid hydrolysis	4.76	2.39	0.210	0.50
Aminosol, for intravenous injection, with acid hydro- lysis	4.62	3.46	0.348	0.75

Identification of the Free Amino Acids.

From the foregoing it will be seen that the majority of the amino acids in casein occur in free form in Aminosol. For the identification of these free amino acids, paper chromatography, a method elaborated by CONSDEN, GORDON and MARTIN (1944), was used in many of the tests. The present author adopted a modification of this technique devised by EDMAN (1945). With the use of this method it was possible to identify most of the amino acids in the casein digest. Some of the amino acids, however, could be identified with a greater degree of certainty, and with greater facility, by another method, partly because their R_F values¹ lie very close to one another in the solvents used in these tests, and partly because there is considerable variation in the concentrations of the different amino acids. Both these factors cause the various spots on the paper to become confluent. In some instances it was more convenient first to isolate the amino acids by chemical methods or by electrodialysis and then to identify them by paper chromatography. In other cases it was easier to identify the amino acids by chemical methods only.

¹ $R_F = \frac{AB}{AC}$: AB is the distance that the amino acid has moved from the point of application and AC the distance that the liquid front has moved from the same point.

Identification of Tyrosine, Tryptophane, Methionine, Valine, Phenylalanine, Isoleucine, Leucine, Proline, Glutamic Acid, and Asparaginic Acid by Paper Chromatography.

These tests were performed with the apparatus described by CONSDEN, GORDON and MARTIN (l. c.). The solvents used were firstly a mixture composed of 35 per cent pyridine, 35 per cent amyl alcohol, and 30 per cent distilled water, and secondly a mixture with a composition of 45 per cent isobutyric acid, 45 per cent isovaleric acid and 10 by vol. per cent distilled water (EDMAN l. c.). The paper used was No. OB, size 50×50 cm.¹ As a reference solution in the chromatographic tests, I used a mixture of known amino acids consisting of

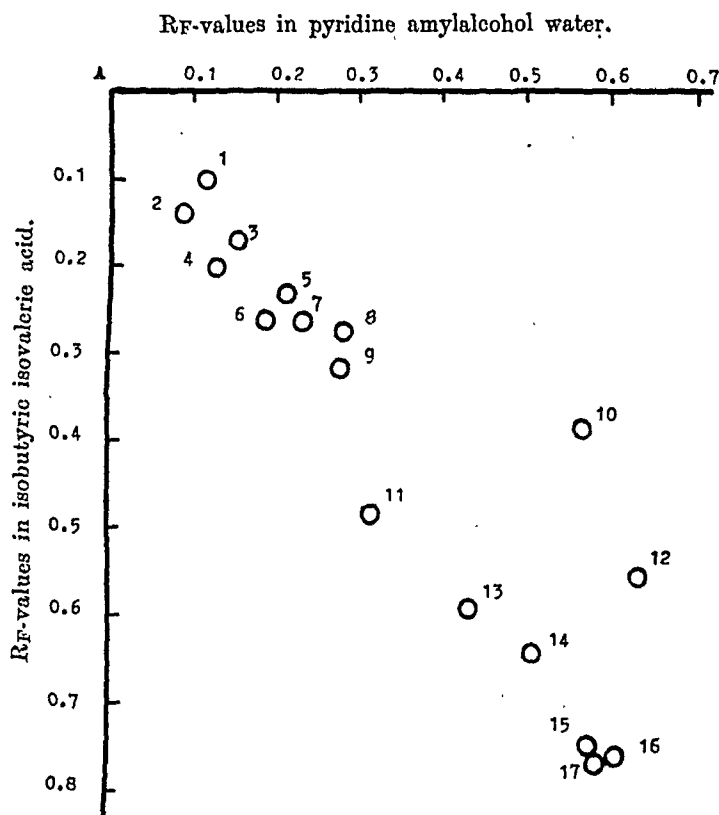


Fig. 1. Paper chromatogram of a mixture of known amino acids. The R_F-values in the solution containing pyridine, amylalcohol and water are plotted on the abscissa, and the R_F-values in the mixture composed of isobutyric acid and isovaleric acid on the ordinate. A denotes the point of application of the amino acid mixture. The dots indicate the position of the various amino acids after the test. 1, cystine. 2, asparaginic acid. 3, lysine. 4, glutamic acid. 5, glycine. 6, arginine. 7, histidine. 8, hydroxyproline. 9, alanine. 10, tyrosine. 11, proline. 12, tryptophane. 13, valine. 14, methionine. 15, isoleucine. 16, phenylalanine. 17, leucine.

¹ From J. H. Munktells pappersfabriksaktiebolag, Grycksbo, Sweden.

glycine, alanine, valine, leucine, isoleucine, serine, threonine, cystine, methionine, proline, hydroxyproline, phenylalanine, tyrosine, tryptophane, lysine, arginine, histidine, asparaginic acid, and glutamic acid. Each amino acid in this mixture was present in a concentration of 1 pro mille. This amino acid mixture was then analysed by paper chromatography. Pyridine, amylalcohol, and water were used as the first solvent and, after drying the paper, isobutyric acid, isovaleric acid, and water were used as solvent in the perpendicular direction. The solvent was run over the paper for 10—11 hours in each direction, the liquid front moving about 40 cm in this time. The chromatographic tests were made at room temperature. Since the R_F values were known (EDMAN l. c.) the various spots were readily identified, especially as the different amino acids yield different colour tones with ninhydrin, which was used to make the amino acid spots visible. Figure 1 shows a chromatogram of known amino acids.

The dialyzed casein hydrolysate was then analyzed by chromatography in the manner described above. The concentration of the casein digest was 3.3 per cent, which corresponds to an approximate concentration of 1 pro mille for the majority of the amino acids. The chromatographic tests were performed on Aminosol preparations intended for peroral and for intravenous administration. Identical chromatograms, shown in Figure 2, were obtained. The spots of the amino acid which did not move further than is indicated by the broken lines in the Figure cover one another, and were therefore impossible to identify. On the other hand, as may be seen from Figure 2, the following amino acids were plainly distinguishable, viz. tyrosine, tryptophane, methionine, valine, phenylalanine, isoleucine, and proline.

The above-mentioned solvents are not suitable for the chromatographic demonstration of asparaginic acid and glutamic acid. On the other hand, phenol saturated with water is a good medium for the purpose, as the R_F values for these two amino acids, in contrast to other amino acids, are very small in this solvent. The chromatographic tests were made in one direction only, on a strip of paper about 10 cm wide. An aqueous solution of asparaginic acid and glutamic acid, in a 1 pro mille concentration for each, was used for purposes of comparison. The concentration of the casein digest was 3.3 per cent. In order to avoid the discoloration and distortion which occurs at the liquid front, the following procedure was adopted. Before the solution of known amino acids and the casein digest were applied to the paper the phenol and water solution was allowed to diffuse about 10 cm.

The two Aminosol preparations, for peroral and intravenous alimentation, yielded the same chromatogram. The result of the test showed that both asparaginic acid and glutamic acid occur in free form in the dialyzed casein digest.

Chromatographic Identification of Lysine and Arginine after Electrodialysis.

An apparatus such as that mentioned by HAMMARSTEN, ÅGREN, HAMMARSTEN, and WILANDER (1933) was used for the isolating of the basic amino acids, arginine and lysine. As membrane, parchment paper was used at the cathode and real parchment at the anode.

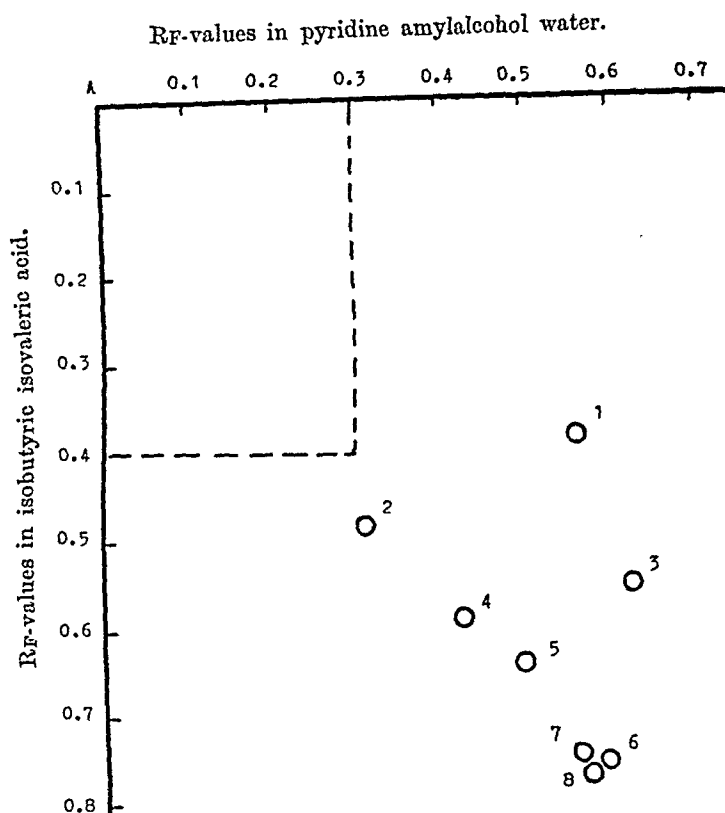


Fig. 2. Paper chromatogram of Aminosol. Same system of notation as in Fig. 1. Amino acid spots in the upper left corner marked off with broken lines were unidentifiable. Other amino acids are 1, tyrosine. 2, proline. 3, tryptophane. 4, valine. 5, methionine. 6, phenylalanine. 7, isoleucine. 8, leucine.

25 ml. of an 8 per cent solution of the casein (the two preparations intended for peroral and intravenous alimentation gave identical results) with a pH of 7.1 were electrodialyzed in the above-mentioned apparatus at a temperature of 0°—+ 4° C, using an amperage of 0.10 Amp. The temperature in the mid-cell was maintained by means of a spiral glass tube through which alcohol cooled by solid carbon dioxide was kept circulating. During the two hours the experiment was in progress 300 ml. of fluid were obtained at the cathode; these were concentrated in vacuo to 20 ml.

Chromatographic analysis, in one dimension, of this concentrated cathode fluid, using pyridine, amylalcohol, and water as the solvent and a solution composed of lysine, arginine, and histidine as a reference fluid, yielded a chromatogram, which showed that arginine and lysine were present in the free form.

The concentration of the various amino acids in the reference solution was 1 pro mille.

Identification of Histidine by Chromatographic Analysis after Silver Precipitation.

As histidine is the least alkaline of the basic amino acids it is presumably not dialyzed out into the cathode fluid during the above-mentioned electrodialysis. Silver precipitation was therefore carried out by the KOSSEL method (BLOCK and BOLLING 1945), in order to identify the histidine, and one-dimensional chromatography was then done.

To 30 ml. of 3 per cent casein digest 50 per cent AgNO_3 was added until one drop of the solution gave a dark brown deposit with diluted $\text{Ba}(\text{OH})_2$. A saturated solution of $\text{Ba}(\text{OH})_2$ was then added until a distinctly blue colour was obtained with bromthymolblue paper. The precipitate was centrifuged, washed with water and suspended in 20 ml. of water, and sulphuric acid was added until a Congo-positive reaction was obtained. The silver was then precipitated with hydrogen sulphide. The solution was filtered and then subjected to one-dimensional chromatography with pyridine, amyl alcohol, and water. A 1 pro mille histidine solution was used as the reference fluid.

It was shown that histidine was present in the free form. The same result was obtained both from the peroral and the intravenous Aminosol preparation.

Identification of a Number of Acids by Chemical Methods. Threonine.

A method suggested by NICOLET and SHINN (1941: 138), by which the threonine is oxidized to acetaldehyde by periodic acid was used for analysing the threonine. The acetaldehyde was distilled into bisulphite and determined by titrimetry.

For the determination, 0.2584 g and 0.2458 g of the casein digest for peroral alimentation were used, and amounts of 2.97 and 3.08 per cent respectively of threonine were obtained. When the preparation for intravenous alimentation was used, 2.6 per

cent of threonine was obtained. In the latter determination 5.00 ml. of 3.3 per cent solution were employed.

Serine.

The serine was determined by the NICOLET and SHINN method (1941: 139). The principle of this method is that the serine is oxidized to formaldehyde with periodic acid. After the acetaldehyde simultaneously formed from the threonine had been distilled off, the formaldehyde was determined by precipitation with dimethyldihydroresorcinol (dimedon).

0.2541 g of the peroral casein digest gave a precipitate with dimedon weighing 16.7 mg corresponding to 6.01 mg of serine. The serine content was therefore 2.36 per cent. The melting point of the dimedon derivative was 188° C.

5.0 ml. of the casein digest for intravenous use gave precipitation with dimedon weighing 26.8 mg corresponding to 5.9 per cent serine.

Alanine.

The free amino acid, alanine, was determined by a new micro-method elaborated by ÅQVIST. With this method, alanine is deaminized to lactic acid with nitrous acid, after which the lactic acid is determined by colorimetry with p-hydroxydiphenyl by the MILLER and MUNTZ (1938) method. This reaction is not given by any other amino acid. The amount of alanine in the casein digest thus determined was 2.0 per cent, in the peroral as well as in the intravenous preparation.

Summary.

In a dialyzed casein hydrolysate (Aminosol), produced by the hydrolysis of casein with trypsin and erepsin 76 per cent of the peroral preparation and 67 per cent of the intravenous one is composed of free amino acids.

In this dialyzed casein digest the following amino acids occur in a free form viz. alanine, arginine, asparaginic acid, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, valine.

The investigation showed that the ten essential amino acids,

arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine, occur in the free form, a fact which, chemically speaking, proves that this dialyzed casein digest is an adequate source for the nitrogen needs of the body.

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From the Physiological Institute, University of Uppsala.

The Mode of Entrance of Sodium into the Aqueous Humour.¹

By

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KINSEY and GRANT (1942) have shown that the dynamics of the accumulation of various electrolytes from the blood in the aqueous humour of the rabbit's eye can be explained on the assumption that the rate of entrance is proportional to the concentration in the blood but independent of the concentration in the aqueous humour itself. They consider this proof that electrolytes enter the eye by secretion. Pure ultrafiltration, however, with negligible back diffusion would give the same relationship. Therefore their work only shows that electrolytes do not enter the aqueous humour by diffusion.

It thus remains to be determined whether electrolytes enter by ultrafiltration (with negligible diffusion) or by secretion. In order to decide between the two alternatives, the influence of arterial blood pressure on the rate of entrance of radioactive sodium (Na^{24}) has been studied. For ultrafiltration to be possible against intraocular pressure and colloid osmotic pressure of the blood a rather high capillary pressure is required. But a high capillary pressure would follow variations in the arterial pressure closely since there seems to be no mechanism stabilising the capillary pressure of the eye when the arterial pressure drops (BÁRÁNY 1945, 1946, 1947 a, b, 1947 a, b). Therefore a material decrease in

¹ The radio-active sodium used in the experiments was kindly furnished by Prof. Manne Siegbahn, Research Institute for Physics. The investigation was aided by a grant from the Lennander Fund of the University of Uppsala.

arterial pressure would materially reduce the filtering capillary pressure and thereby the rate of ultrafiltration. If sodium enters by ultrafiltration, the rate of entrance should be reduced by a drop in blood pressure.

Methods.

Rabbits with a van Leersum carotid loop were used. The arterial blood pressure of the one eye was reduced by clamping the loop 2—3 hours before the experiment. The blood pressure difference between the two sides of the head was estimated with a pressure capsule applied to the heat dilated central arteries of the ears at least 30 minutes after clamping of the loop. (BÁRÁNY 1946 a.)

All animals irrespective of body weight were injected intraperitoneally with 5 ml 0.9 % NaCl containing about $24 \cdot 10^6$ counts per minute. They were anaesthetized with Numal "Roche" (allyl-isopropyl-barbituric acid), in some cases intravenously shortly before the withdrawal of aqueous, in some cases intraperitoneally, about 1.5 hours before the aqueous sampling. The Numal was always diluted with a few ml of normal saline.

Aqueous was withdrawn by means of stainless steel cannulae, outer diameter 0.5 mm. The aqueous was drawn by gentle suction into whole-glass syringes. The tips of the cannulae were very carefully ground under a binocular microscope previous to every puncture. A really sharp cannula usually enters through the cornea with very little resistance. In order to avoid air bubbles, the junctions between cannulae and syringes were bandaged with adhesive tape and the plungers of the syringes were oiled with pure vaseline. The syringes were mounted with a stop arrangement, set at 0.25 ml.

For withdrawal of aqueous, the lightly anaesthetised animal was laid on a table with all limbs perfectly free to move. An assistant fixed the head by means of a firm grip around the nasal bones and the skull. With these precautions, even rather violent movements of the animal are possible without serious displacement of the cannula in the eye. It is not necessary to luxate the eye, as is usually recommended (see for instance VELHAGEN 1937), if one fixes the bulb by means of a pin-clip placed on the tendon of the superior rectus muscle. The puncture should be a corneal puncture, a few mm inside the limbus. A limbal puncture in the rabbit easily results in lesion of the iris.

Orientation in the shallow anterior chamber of the rabbit is much facilitated by the use of a binocular loupe (x 2) and this was used in all experiments. Experiments where the iris was macroscopically injured or even where it showed contraction during the few seconds of aspiration were discarded.

Blood for the plasma samples was taken by gentle suction from a marginal vein of the ear and passed directly into a centrifuge tube containing dry heparin.

Radio-sodium was determined in 0.1 ml samples. All activities in the following table refer to this volume and are corrected for radioactive decay. The half-life of Na^{24} was taken as 14.8 hours.

Results.

The results of the experiments are summarized in table I.

The table is largely self-explanatory. Column 5 shows the really relevant figures: the ratio of activity between the aqueous of the occluded and the control side. As is seen, the ratio varies somewhat around its mean, 0.961. The two extreme values 0.719 and 1.219 happen to deviate about equally much from 1.0. This is of course a coincidence as probably both are the results of some kind of error. For the value 1.219 it is possible that the accident during withdrawal of control aqueous may account for the deviation. The other extreme value was, however, obtained from a perfectly satisfactory puncture and there is nothing in the further experiment to account for the deviation. If all experiments with some hitch or other during the aqueous sampling are discarded, the remaining 4 give a mean ratio of 0.91. The experiments thus show that carotid occlusion has but a small influence.

In column 11 a series of figures is given showing the degree of distribution of the injected Na^{24} over the body. As the same amount of radio-sodium was injected into all animals, the degree of dilution should be proportional to the total sodium space of the body, and thus to the body weight. Therefore, after equilibrium is established, the product of concentration of Na^{24} , expressed in counts per minute, and body weight, in kilograms, should be approximately constant. The column shows this to be the case for all animals with the exception of nr 610. Here, only about half the usual concentration was reached. Probably the injected material was deposited in the intestine instead of the peritoneal cavity.

Discussion.

The nearly equal aqueous concentration on the occluded and control side may be thought to indicate that, at the moment of aqueous withdrawal, both eyes had already come into equilibrium with the blood in respect to Na^{24} , or that the rise in plasma concentration, which of course is equal for both sides, had been the

Table 1.

Date	Animal n:r	Aqueous					Plasma		Mean blood-pres- sure in ears, mm Hg.		Weight of animal kgm. \times counts \times 10 ⁻³	Anaesthesia, ml Nungal/ kgm	Remarks
		Counts per minute		Time of withdrawal after injec- tion, minutes Occl.-Cont.	Counts per minute	Time of with- drawal after in- jection, minutes	Occluded	Control					
		Occluded	Control										
									Occluded	Control			
1	2	3	4	5	6	7	8	9			10	11	12
30/1 . .	628	1,535	1,610	0.955	41 —42	3,830	236	53.1	65.6	9.2	0.6 i.v.	a)	
30/1 . .	613	411	572	0.719	47 —48	3,420	240	34.4	65.0	8.2	0.6 i.v.		
30/1 . .	631	2,278	2,440	0.934	52 —53	4,900	243	38.6	62.6	9.8	0.6 i.v.		
31/1 . .	604	1,765	1,450	1.219	44 —45.5	4,310	34	52.3	66.3	8.2	0.63 i.p.	b)	
31/1 . .	610	890	867	1.025	52.5—51	2,240	38	54.1	72.5	4.5	0.62 i.p.		
31/1 . .	592	1,101	1,200	0.919	58.5—59.5	3,360	41	62.1	75.8	8.4	0.64 i.p.	c)	
31/1 . .	593	1,560	1,670	0.934	61.5—62.5	3,710	50	39.8	75.8	9.7	0.62 i.p.	d)	
31/1 . .	636	2,490	2,540	0.981	66.3—67.3	4,530	46	46.3	62.5	9.6	0.60 i.p.	e)	
	Mean			0.961				Mean difference: 20.6					
								Mean ratio: 0.70					

a) Withdrawal of control aqueous rather slow, the syringe sticks.

b) Withdrawal of control aqueous: The tip of the cannula was in the cornea but had not perforated when the animal began to jerk for some seconds. The cannula was not withdrawn, however, and after the jerks ceased the puncture was completed. Some massage of the bulb certainly occurred during the jerks.

c) Corneal puncture on occluded side met with unusual resistance.

d) During aspiration of occluded side the animal sneezed once but the aspiration was completed in a few seconds.

e) The anaesthesia had to be completed with 1 drop of 0.4 % Diocain into each eye 2 minutes before the aspiration of the aqueous. Otherwise: perfect.

limiting factor. However, the figures just discussed (column 11) show that full plasma concentration was reached already before the aqueous was drawn. There is no significant difference between values obtained several hours after injection and those obtained after 30—50 minutes. This is in good agreement with the curves obtained by KINSEY *et al.* (1942), which show full plasma concentration to be reached already 20—30 minutes after intraperitoneal injection of Na^{24} . Thus the plasma concentration could not have been the limiting factor. Nor can the equilibrium explanation be true. The plasma figures in column 7 are at least twice as large as those for aqueous shown in column 3 and 4. As the equilibrium concentration of sodium in the aqueous would be about 90 % of that of the plasma, this shows that at the moment of withdrawing aqueous, the radio-sodium concentration of the aqueous was still rising. This, too, is in good agreement with the curves of KINSEY *et al.*, which show definitely rising aqueous concentrations several hours after intraperitoneal injection of Na^{24} .

It follows that our concentration values are an expression of *accumulation rates* and that consequently our experiments show that the rate of accumulation of Na^{24} in the aqueous is very little influenced by clamping of the carotid.

This finding would be consistent with the ultrafiltration hypothesis only if the drop in filtering pressure caused by carotid clamping were negligible. A rough calculation shows, however, that this could not be the case.

Using a micro-injection technique, DUKE-ELDER (1926) found a mean pressure of about 75 mm Hg in the retinal arteries of the cat shortly after their entrance into the eye. SEIDEL (1924), by compression of the anterior ciliary arteries in man, came to a mean pressure of 42—60 mm Hg for the arteries of the iris and ciliary body. If we take the pressure at the beginning of the arterial limb of the filtering capillaries to be 80 mm Hg we have certainly not assumed too low a value. This filtering pressure is opposed by the intraocular pressure of 25—30 and the colloid-osmotic pressure of the plasma, 20—25 mm Hg. Assuming the sum of these latter to be 50 the maximum possible effective filtering pressure in the capillaries would become 30 mm Hg.

Clamping the carotid usually reduces the mean pressure in the ophthalmic arteries from the normal value of 100 to somewhat less than 70 mm Hg. The reduction on an average is propor-

tionally as large as the reduction in ear artery pressure. (BÁRÁNY 1946 a.) In the present series the mean ratio between the ear artery pressures was 0.70 (columns 9,10) and this corresponds exactly to a reduction of the ophthalmic artery pressure from 100 to 70 mm Hg. The corresponding pressure drop in those vessels which have a pressure of 80 mm Hg would be at least 20 mm Hg. Clamping of the carotid causes an intraocular pressure drop of about 4 mm Hg (BÁRÁNY 1946 a, b). Thus the maximum filtering pressure would be about $(80-20)-(50-4) = 14$ mm Hg with carotid occluded as against 30 mm Hg with carotid open. The filtering pressure, consequently, would be reduced to less than half the normal value. As a matter of fact, the reduction is certainly even greater, since the assumed capillary pressure of 80 mm Hg is definitely too high and the relative reduction in filtering pressure increases with decreasing initial pressure.

It follows that carotid clamping would have materially reduced the ultrafiltration rate if ultrafiltration actually took place. As it did not greatly affect the rate of accumulation of sodium, either the entrance of sodium does not depend on ultrafiltration or the rate of secretion is increased by the blood pressure drop to a degree almost exactly compensating the reduction in ultrafiltration. This latter alternative is, of course, extremely improbable and contrary to all ordinary physiological experience. The opposite is much more probable: that some reduction in the rate of secretion takes place as a consequence of the reduced blood flow. This reduction of the secretion could explain the fact that the rate of accumulation of sodium was a little lower on the side of the occluded carotid.

Summarizing, then, it is most probable that the entrance of sodium does not depend on ultrafiltration, and that sodium enters the aqueous mainly or wholly by secretion.

Summary.

The rate of accumulation of radio-active sodium in the aqueous humour of the rabbit is not appreciably influenced by the blood pressure reduction caused by occlusion of the common carotid artery. If sodium had entered the aqueous by ultrafiltration, a material reduction of the rate of entrance would have occurred. Consequently sodium does not enter the aqueous by ultrafiltration but mainly by secretion.

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On the Function of the Pineal body.

By

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The metabolism of the brain has long been the subject of comprehensive research. A possibility of analysing this metabolism under more physiological conditions than those hitherto employed has been offered by the introduction of the marked isotopes, especially the radioactive isotope of phosphorus, into biological investigations. This procedure permits a study of the metabolism in the living organism in a manner which has previously been impossible.

Using this procedure, BORELL and ÖRSTRÖM (1945) showed that, of seventeen parts of the brains of rabbits or rats, the pineal body showed the highest phosphate turnover. This surprising discovery has since been confirmed in the cases of the cat, the dog and the pig.

In order to exclude the possibility that the high phosphate turnover of the pineal body might be due to a large admixture of blood with the organ, the blood in the brain of a rabbit was rinsed out after the slaughter of the animal by the injection of 200 ml. distilled water in to the carotid arteries. In spite of this procedure, the pineal retained its high activity (BORELL and ÖRSTRÖM 1947).

Analyses of different phosphate fractions showed, moreover, that 60 % of the radioactive phosphate penetrating into the organ was recovered from those fractions which were associated with the carbohydrate metabolism. Within the pineal the larger part of the added inorganic phosphate had rapidly been converted to organically bound phosphate.

This demonstration of the high phosphate turnover in the pineal body was very perplexing. Even the pituitary body, with its high production of hormones, did not show a turnover of an equal magnitude.

By means of the current methods employed for the analysis of the hormonal function of an organ, such as extirpation, extraction and feeding, it has not been possible to demonstrate with certainty a hormonal function in the epiphysis. At present, indeed, the function and significance of this organ are completely unknown.

In an attempt to throw some light on this problem we have investigated the specific activity of the epiphysis at different ages and after castration, and changes in the specific activities of different organs after epiphysectomy. 42 different organs or systems of organs have been studied in this manner.

Method.

Rats have principally been employed as test animals.

A small amount of radioactive phosphorus (0.02—0.03 millicurie) was injected intraperitoneally. Forty minutes later the animal was decapitated and the organs analysed for phosphate content and radioactivity (for experimental details, see BORELL and ÖRSTRÖM 1945).

Organs from three animals were united in order to give measurable amounts of phosphate in view of the smallness of the organs that were frequently concerned. All values are given as specific activities. This involves calculation of the number of counts per microgram P:

$$\frac{\text{No. of counts}}{\mu\text{g. P}} = \text{Specific activity.}$$

The higher the specific activity is, the more rapid has been the turnover of the marked phosphate atoms and the higher the metabolism of the organ in question.

With regard to the procedure employed in pinealectomy, it may be mentioned that we operated on rats weighing 50—100 g. Avertin, administered intraperitoneally, was used as anaesthetic. By means of a dentist's drill an opening of about 1 sq. cm. was made in the bone above the region where sinus sagittalis enters sinus transversus. The diffuse bleeding which occurred when the bone was removed was easily stopped with the aid of a cotton swab. A small opening was made in the dura about $\frac{1}{2}$ cm. laterally from the lower part of sinus sagittalis near the point where the latter opens into sinus transversus. A fine haemostat was introduced into the hole, by means of which sinus transversus was constricted on one side. With an electric cautery the sinus transversus, laterally from the haemostat, and the bordering Barr-

of the dura were burnt off without damage to the brain. The haemostat was then carefully turned medially, which revealed the pineal, of the size of a pinhead, lying in the angle between sinus sagittalis and sinus transversus. The pineal body was then removed with forceps. The resultant bleeding was stopped by gentle pressure with cotton-wool. The extirpated bone was restored to position and the skin was sutured. By means of this procedure only the sinus transversus on one side is damaged while the other large veins in the animal remain relatively uninjured.

The animals were injected with radioactive phosphorus, usually 5—9 weeks after the operation but in individual cases up to 9 months afterwards. When the animals were being killed through decapitation a small injury was frequently observed on the corpora quadrigemina as a consequence of the operation. In all about 50 animals were operated upon. The mortality in connection with the operation was about 50 % in the first series, but subsequently diminished appreciably. Thus, in the final series of 14 animals all the individuals survived.

Results.

The phosphate metabolism in the pineal from animals of different ages is shown by curve 1. The animals were given about 0.020 ml per 100 g. body weight. Some further brain parts which were of interest in this connection were also investigated, namely the tuber cinereum, the adenohypophysis and the neurohypophysis. For the purpose of including a part without a hormonal function, the cerebellum was also studied. The animals were of the approximate ages 5, 10, 52 and 104 weeks. The groups contained 24, 36, 11 and 13 animals respectively. These ages represent different developmental stages. At the age of 10 weeks the vagina commences to open, while at 65 weeks the menopause begins (see "The Rat" page 3). Our groups of animals thus included juveniles, individuals in the period of transition to fertility, fertile and senile rats. Both males and females were investigated. The activity in the epiphyses from females was usually somewhat higher than in the case of males, although it was impossible to establish any certain difference.

The high phosphate turnover in the pineal body at all ages is clearly shown by the curve. Its turnover is 2—3 times as great as that in the adenohypophysis. The curve for the pineal indicates a sharp rise in the activity during the first months after birth, while the value remains relatively constant during the fertile period. In senile animals a further increase in activity occurs.

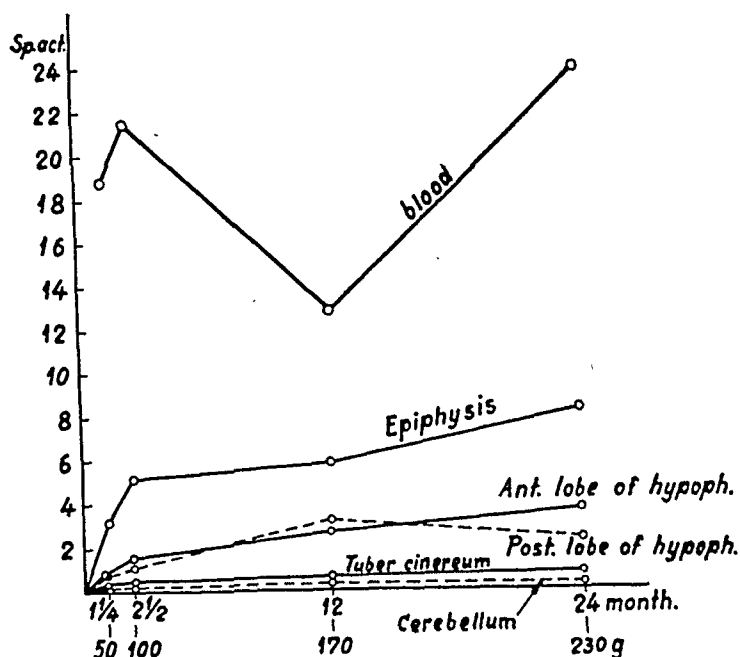


Fig. 1.

Specific activity of P³² in different brain parts from rats of different ages.

The curve shows that the epiphysis has the lowest metabolism during the infantile period, which fact is to a certain extent contrary to previous conceptions of the function of the pineal during growth. It has been considered, indeed, that the task of the pineal is to exert an inhibitory action on the sexual maturation. We have, however, found the highest phosphate turnover in the pineal from fertile and senile animals. As will subsequently be shown, we also found that the pineal has a stimulating action on the ovaries.

The phosphate turnover in the adenohypophysis has essentially the same course as that in the epiphysis. It shows the lowest values during the first 75 days of the life of the animal. In the fertile period the activity is high but relatively constant, ultimately increasing somewhat.

The curve for the posterior lobe proceeds largely in the same manner as that for the anterior lobe. In old animals the values are somewhat lower. The activity in the tuber cinereum changes in a manner analogous to that of the adenohypophysis, although the values are lower throughout.

The activity of the cerebellum does not change with age. That of the blood, which constitutes a rough measure of the amount of radioactive phosphate injected, may vary in a high degree within the concentration range employed by us without affecting the activities of the brain parts investigated.

In order to determine whether the specific activity shows variations parallel with the phosphorus content in the organs from rats of different ages, we have assembled the relevant data in Table 1. The values are given in $\mu\text{g. P}$ per organ. An increase in the specific activity proves to be not necessarily accompanied by a corresponding change in the phosphate content. The table shows that in the two youngest age groups the phosphate content remains practically unaltered in the brain parts investigated, despite that the specific activity increases in all of these with the exception of the cerebellum.

Table 1.

Phosphate contents of different brain parts from rats of various ages.

Age group	$\mu\text{g. P}$			
	Pineal body	Adeno-hypophysis	Neuro-hypophysis	Cerebellum
1½ months (40—70 g.)...	16.5	35.8	24.5	1,750
2½ months (70—130 g.)..	14.8	34.9	24.7	1,655
12 months (130—200 g.) .	18.5	48.1	18.3	2,070
24 months (> 200 g.)....	20.6	65.4	35.2	2,300

The next phase in our investigation comprised the experiments in pinealectomy. The results of these are assembled in Table 2. Each value represents at least 12 and at most 21 animals. A number of organs have been excluded from the table, such as bones, skin, musculature and digestive tract, since the results for these were negative and proved to be devoid of interest in this connection.

The changes in the phosphate metabolism of the brain brought about as a consequence of pinealectomy may conceivably be classed in two groups:

1. Changes that result from injuries incurred in the operation. The parts which may be concerned here are habenula, corp. quadr. and cerebellum. Of these corp. quadr. has been most changed, its activity having been increased from 0.18 to 0.32, or almost doubled. The difference of 0.14 is not statistically significant, with $t = 1.90$, giving $p < 0.1$. The change in this part of the

Table 2.

Effect of pinealectomy on the phosphate turnover (expressed as specific activity) of various organs in the rat.

	Control animals			Epiphysectomized animals		
	Males	Females	Mean	Males	Females	Mean
Habenula	0.63	0.61	0.62	1.13	0.71	0.84
Thalamus	0.21	0.28	0.25	0.23	0.29	0.27
Subst. perf.	0.28	0.24	0.26	0.41	0.88	0.67
Corp. mam.	0.27	0.44	0.36	0.36	0.43	0.40
Corp. quadr.	0.16	0.20	0.18	0.32	0.32	0.32
Pons	0.19	0.20	0.20	0.20	0.23	0.22
Medulla	0.20	0.49	0.35	0.40	0.38	0.39
Ant. tuber cin.	0.39	0.33	0.36	0.27	0.40	0.37
Post. tuber cin.	0.28	0.33	0.31	0.22	0.29	0.28
Centr. tuber cin. ...	0.48	0.58	0.53	0.51	0.85	0.68
Adenohypophysis ...	1.87	2.00	1.95	2.95	3.50	2.96
Neurohypophysis ...	2.14	2.95	2.55	1.67	3.95	3.33
Cerebellum	0.25	0.28	0.27	0.31	0.38	0.35
Plex. chor.	11.00	1.85	6.22	2.85	6.05	4.90
Thyroid	15.30	14.15	14.65	15.65	16.20	16.00
Thymus	4.58	5.06	4.85	5.75	6.60	6.35
Spleen	7.42	8.66	8.10	4.83	7.58	6.60
Liver	23.70	10.55	14.90	13.80	15.10	14.48
Adrenal gland	7.36	6.72	6.98	6.62	8.85	8.03
Bone marrow	6.65	4.20	5.43	3.70	4.40	4.15
Ovaries	—	17.45	—	—	10.09	—
Uterus	—	17.10	—	—	8.10	—
Prostata	14.94	—	—	10.33	—	—
Ves. sem.	8.28	—	—	9.58	—	—
Epididymis	8.08	—	—	8.60	—	—
Testis	2.25	—	—	3.80	—	—
Blood	18.05	19.65	18.85	13.60	19.75	17.93

brain is the same in males and females. The other three parts also show differences, but these are smaller and not statistically significant. The spreading of the values is very great in the cases of habenula.

2. Changes that probably arise as a result of the removal of the pineal. These changes are different in males and females.

The organs in which the phosphate turnover changes most appreciably in females are especially the uterus, followed by the ovaries, adenohypophysis, neurohypophysis and tuber cinereum, while only the prostata are subject to considerable change in males. The large differences occurring in the table that are not discussed in the text, *e. g.* in substantia perforata, liver and plexus chorioideus, are due to the occurrence of one highly divergent value which has a great effect upon the mean.

The specific activity of the anterior lobe rises in females from 2.00 to 3.50 after pinealectomy, that of the posterior lobe from 2.95 to 3.95 and that of the central part of the tuber cinereum

from 0.58 to 0.85. No great changes are observed in these parts of the brains of males. In no case, however, is the increase statistically significant. The activities of the ovaries and the uterus after pinealectomy show a behaviour opposite to that of the brain parts. Their activities fall from 17.45 to 10.69 and from 17.10 to 8.10 respectively. This later value, with $t = 1.94$, is not statistically significant. The difference of 6.76 in the specific activity of the ovaries is statistically probable, with $t = 2.67$ and $p < 0.05$. In males there is a decrease in the activity of the prostata from 14.94 to 10.33, but this difference is not statistically certain in view of the great spreading within the group. The same is true for the rise of the specific activity in the testis.

Discussion.

The most striking changes after pinealectomy occurred in the turnovers of the uterus and the ovaries, which rapidly decreased after this operation. It hence appears probable that the epiphysis exerts a stimulating effect upon the sexual organs of the female. Whether this takes place directly or indirectly cannot be decided at the present stage.

In the investigation of the pituitary body it has been shown that this gland not only affects the ovaries but is also affected in a high degree by the hormones of these organs. This has been shown by, among other things, castration experiments. Analogously, it is clearly of interest to determine whether castration also influences the pineal. We therefore castrated 6 male and 8 female rats and measured the phosphorus turnover of the pineal body at least six weeks after the operation.

The results are collected in Table 3. It will be observed that the phosphate turnover in the pineal rises in males after castration from 6.20 to 7.60, while the corresponding increase in females is from 9.65 to 15.72. Thus, as in the results of the pinealectomy experiments, the changes are greater in females. In the adeno-hypophysis also an increase in specific activity occurs in both males and females, although it is more marked in the latter. This organ is known to give off increased quantities of gonadotropine after castration (ENGLE 1929, and LAUSON, GOLDEN and SEVERINGHAUSEN 1939). This increase hence takes place parallel with a rise in the phosphate turnover. It does not appear impossible

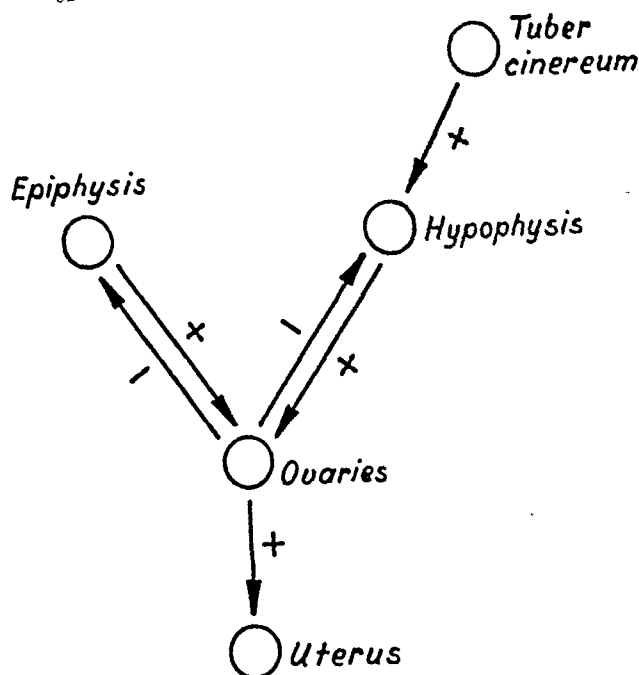


Fig. 2.

that, in the case of the epiphysis also, the phosphate turnover is an indicator of a hormone synthesis and that there is a hormonal interchange between the epiphysis and the sexual organs, especially of the females.

Table 3.

Effect of castration upon the phosphate turnover of the pineal body and the adenohypophysis (expressed as specific activity).

	Males		Females	
	Epiphysis	Adeno-hypophysis	Epiphysis	Adeno-hypophysis
Controls	6.20	1.87	9.65	2.00
Castrates	7.64	2.00	15.72	3.12

The following scheme (Fig. 2.) gives a conceivable explanation for these phenomena and is in concordance with the experimental results.

The pineal activates the ovaries and directly or indirectly the uterus. It is of interest in this connection that certain workers, including SILBERSTEIN and HAGEL (1933) and SAPHIR (1934), claim to have found oestrogenic substances in the pineal, which result, however, TARKHAM (1937) and WADE (1937) have been unable to confirm. Conversely, the ovaries exert an inhibitory action upon

the epiphysis. An analogous relationship prevails between the adenohypophysis and the ovaries, although the substances which regulate the interchange in this case are known. The gonadotropins stimulate the ovaries, while the oestrones inhibit the adenohypophysis. Under certain conditions, such as at coitus, the tuber cinereum is also involved in this interchange (BORELL, WESTMAN and ÖRSTRÖM 1946). In our experiments, especially in the case of females, a rise in the phosphate turnover has been observed in the tuber cinereum after pinealectomy. An explanation of this phenomenon may be that the reduction in the activity in the ovaries after the pinealectomy probably brings about a reduction in the amount of oestroné formed. As a consequence of this, there will be a decrease in the inhibitory action of this hormone on the pituitary-diencephalon system.

The increase in old animals of the phosphate turnover in the epiphysis, adenohypophysis and tuber cinereum (see p. 65) would then be explained by a cessation in the inhibition of the brain parts concerned by the hormones of the ovaries. The activities of these parts would then increase.

The decrease in the phosphate turnover of the uterus after epiphysectomy may be caused secondarily by a primary reduction in the activity of the ovaries.

Summary.

1. The phosphorus turnover of the pineal has been studied with the aid of radioactive phosphorus in rats of different ages and compared with the turnovers of the adenohypophysis, neurohypophysis, tuber cinereum and cerebellum. The activities in these organs with exception of cerebellum are lowest during the first 10 weeks of the life of an animal. In the fertile age the activity remains at a constant high level and with exception of the neurohypophysis increases somewhat during senility. The activity of the cerebellum remains unchanged.

2. Of the 42 organs and parts of the brain investigated, the phosphate metabolism decreased only in the ovaries after epiphysectomy to a statistically probable extent. Considerable increases in the turnover were also observed in the adenohypophyssi, neurohypophysis and tuber cinereum in females.

3. After castration an increased phosphorus turnover was obtained in the pineal body and the adeno-hypophysis. This increase was most pronounced in females.

4. It appears to follow from these results that the pineal exerts an influence, directly or indirectly, especially upon the ovaries. There appear to be reciprocal effects of a similar nature between the ovary and the epiphysis as between the adeno-hypophysis and the ovaries.

We wish to express our thanks to Professor Manne Siegbahn for his kindness in placing radioactive phosphorus at our disposal.

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The Action of β -dimethylaminoethyl Benzhydryl Ether Hydrochloride on Salivary Secretion.

By

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The new antihistamine drug β -dimethylaminoethyl benzhydryl ether hydrochloride is receiving much attention at the present time because of its beneficial action in certain allergic conditions (McELIN and HORTON 1945, EYERMAN 1946, FRIEDLÄNDER and FEINBERG 1946, HARLEY 1946, LEVIN 1946, WALDBOTT 1946). In its clinical application a number of side-effects have been observed and among them is dryness of the mouth. In this paper an account is given of an investigation into the action of the drug on salivary secretion.

The experiments were conducted on cats under chloralose anaesthesia. The blood pressure was recorded in the femoral artery by means of a membrane manometer. The submaxillary duct was exposed and cannulated, and the saliva flow was registered by an electrical drop recorder. The lingual nerve was tied and a Sherrington electrode placed in position just proximal to the point at which the corda tympani branches off. In some experiments a second electrode was placed on the sympathetic nerve in the neck. The two electrodes were connected to a thyatron stimulator. β -dimethylaminoethyl benzhydryl ether hydrochloride² was injected into the femoral vein. The chorda tympani was stimulated in 8 and the sympathetic in 3 experiments. Stimulation times of 1 minute were used, and the gland was allowed to rest for 3 or 5 minutes between each period. In 2 experiments secretion was

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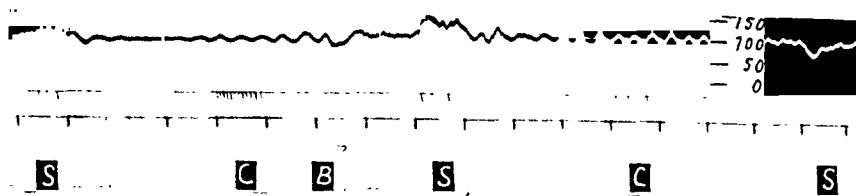
² In this investigation we used "Deseryl Leo" supplied by courtesy of AB Leo, Hålsingborg.

Experiment nr	Rate of secretion, drops/min.		Dose of deseryl mg/kg
	before	after	
1 chorda	12, 10, 12	0, 0, 0	10
2 chorda	12, 12	1, 1, 1	3
3 chorda symp.	11, 12 6, 5	3, 1 3, 0	2
4 chorda symp.	7, 7, 8 10, 10, 9	1, 1, 1 3, 3, 1	3
5 chorda ach, 5 γ	11, 12, 12 5	2, 6, 10 1, 2, 4	3
6 chorda ach, 10 γ	2, 3 14	0, 0, 0 1, 1, 3	3
7 chorda	21, 21	17, 19	1
		16, 17	1
		12, 15	1
		11, 11	2
		1, 1	2
8 chorda symp.	12, 11, 12 7, 8, 10	12, 12, 12	1
		4, 4, 5	10 total
		10, 9 5, 6, 6	1 10 total

² In exp. nr 8 the total dose of 10 mg/kg was given in 6 separate injections and a gradual response was seen as shown in exp. nr 7.

elicited by acetylcholine (5—10 γ) introduced directly into the carotid artery through a hypodermic needle. In 3 experiments intravenous pilocarpine was used.

Results. The results are shown in the table. Deseryl in the doses used clearly depresses chorda, sympathetic and acetylcholine secretion. The threshold dose appears to be 2—3 mg per kg. Repeated doses of 1 mg per kg at short intervals (15—30 minutes) seem to be less effective than a single larger dose. Where inhibition was slight there is evidence of recovery taking place, but where it was more marked the effect seemed to last throughout the experiment. The figure gives an example of the inhibitory effect (experiment nr 3 of the table). A constant rate of secretion produced by pilocarpine was also markedly reduced (in one ex-



The action of β -dimethyl on salivary secretion.

Cat, 2.4 kg, cloralose. Records from above: blood pressure, rate of secretion, time in minutes, signal. S: sympathetic, C: chorda stimulation B: β -dimethyl-aminoethyl benzhydryl ether hydrochloride 2 mg/kg.

periment 20 drops per min. to 12, in the others 10 to 4 and 10 to 3 respectively).

Discussion. From our experiments it appears that β -dimethyl-aminoethyl benzhydryl ether hydrochloride has an atropine-like effect on salivary secretion. It has been shown by LOEW *et al.* (1945) that in certain concentrations this drug antagonises both histamine and acetylcholine on the isolated guinea-pig gut.

The smaller doses which we found to have inhibitory effect are of the same order as those which clinically may cause dryness of the mouth.

Summary.

Experiments on the cat show that β -dimethylaminoethyl benzhydryl ether hydrochloride (2—10 mg/kg) depresses salivary secretion induced by chorda and sympathetic stimulation and by injection of acetylcholine and pilocarpine.

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The Effect of β -dimethylaminoethyl Benzhydryl Ether Hydrochloride on Histamine-Induced Gastric Secretion in the Cat.

By

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Investigations into the problem as to whether histamine plays a rôle in the physiology of gastric secretion would be facilitated by the existence of a substance specifically antagonising the effect of histamine. A great number of substances which counteract the action of histamine on smooth muscle have appeared in the last years. These substances, however, do not seem to exert any marked anti-histamine effect on gastric secretion.

LOEW and CHICKERING (1941) examined the effect of the Fourneau substance thymoxyethyldiethylamine (929 F) on histamine-induced gastric secretion in dogs with Heidenhain pouches. No antagonistic effect was observed; on the contrary, 929 F, without having any stimulating effect itself, increased the secretory response to histamine. Similar results were obtained by BURCHELL and VARCO (1942) and by HALLENBECK (1943). Investigating another Fourneau substance, 1571 F, BURCHELL and VARCO were not able to demonstrate any antihistamine effect, whereas BOURQUE and LOEW (1943) found a slight reduction of the secretion. The antagonism, however, was not specific, as pilocarpine-induced secretion was also diminished to the same degree. Diethylaminoethyldihydroanthracene-carboxylate, another substance with anti-histamine action on smooth muscle, has been found to diminish the volume slightly but not the acidity of histamine-induced gastric juice in dogs (LEHMANN and YOUNG 1945). Pyribenzamine,

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which also antagonises histamine on smooth muscle (YONKMAN, CHESSE, MATHIESON and HANSEN 1946) has according to NECHELES (1946) no influence on gastric secretion induced by injection of histamine.

β -dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) is a new substance with marked antihistamine effect on bronchi and gut (LOEW, KAISER and MOORE 1945). It seems to have a relatively low toxicity and has aroused great clinical interest since it has been found to be of value in the treatment of certain allergic conditions. Some reports regarding its action on histamine-induced gastric secretion have recently been published. McELIN and HORTON (1945) investigated the action of this substance on gastric secretion in man and suggested that benadryl depresses the gastric response to histamine. MOERSCH, RIVERS and MORLOCK (1946) found no reduction in the concentration of free hydrochloric acid and no consistently significant reduction in the total volume of secretion in man. In three out of four dogs LOEW, MAC MILLAN and KAISER (1946) found an inhibition of histamine-induced gastric secretion; in the fourth dog benadryl did not alter the secretory rate. In experiments on four dogs SANGSTER, GROSSMAN and IVY (1946) did not observe any significant effect of benadryl on gastric secretion after histamine.

Experimental.

The experiments were made on 14 cats under chloralose anaesthesia. Blood pressure in the carotid artery was registered using a membrane manometer. The rectal temperature was controlled throughout the experiment. A cannula was inserted into the stomach through an opening in the abdominal wall and the pyloric region was tied. The cat was placed in such a position as to facilitate the outflow of gastric juice. The rate of flow was registered by means of an electrical drop recorder operating a Fleisch "Ordinatenschreiber". This recorded the time interval between the drops as ordinates. In some of the experiments the motility of the gut was registered by means of a balloon placed in the duodenum and connected with a water manometer. Histamine, dissolved in saline, was injected at a constant rate (2—7, usually 2—3 γ per kg of body weight per minute) in a femoral vein. The animals received 0.2—0.8 ml of fluid per kg per minute. Injections of β -dimethylaminoethyl benzhydryl ether hydrochloride¹ (10 mg/kg) were given through a cannula placed in the other femoral vein.

¹ In our experiments we used "Deseryl Leo" which was kindly supplied by AB Leo, Hålsingborg.

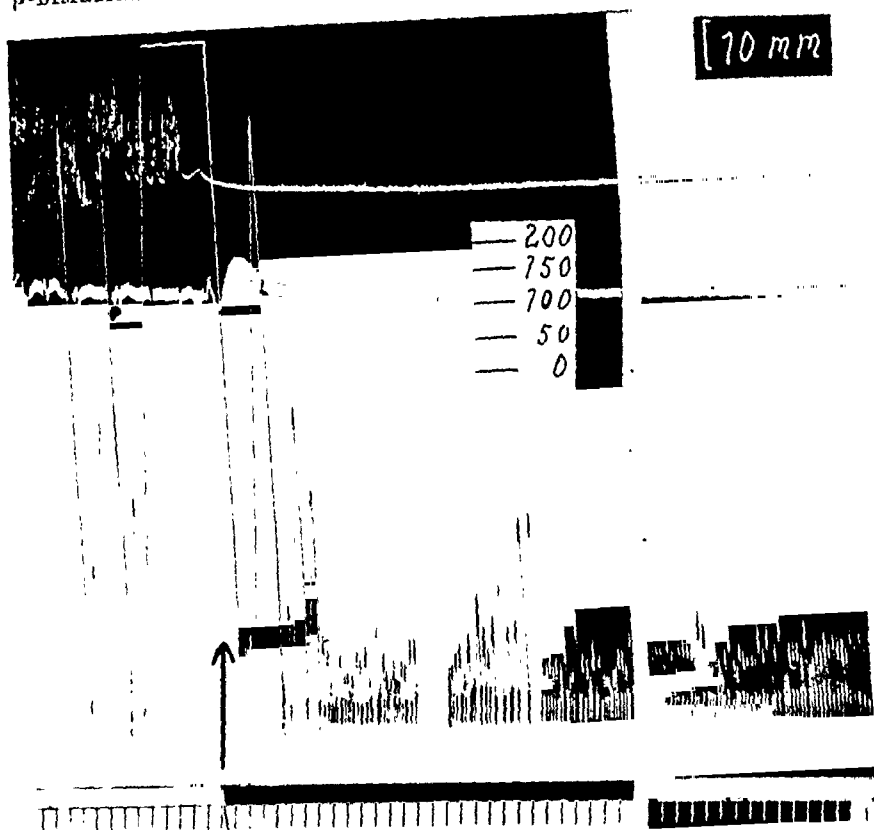


Fig. 1. Cat, 4.0 kg, chloralose. Records from above: 1) motility of the duodenum, 2) blood pressure in the carotid artery, 3) rate of gastric secretion (1 mm of ordinate corresponds to 1 sec. in the original tracing), 4) signal, 5) time in minutes. Between the two sections of the tracing there is an interval of 20 minutes. Throughout the experiment histamine (4 γ /kg/min.) was injected. The arrow indicates the injection of deseryl (10 mg/kg).

Results.

On ten of our cats secretion of gastric juice was elicited by histamine. Fig. 1 shows such an experiment. In none of these experiments did deseryl diminish the gastric response to histamine. On the contrary in all ten animals there was a more or less marked increase in the rate of secretion. This increase, sometimes preceded by a decrease of very short duration, reached its maximum some minutes after the administration of the drug. The rate of secretion seemed to be augmented for a long periode; we have never seen the effect disappear although one of the experiments lasted for as long as five hours.

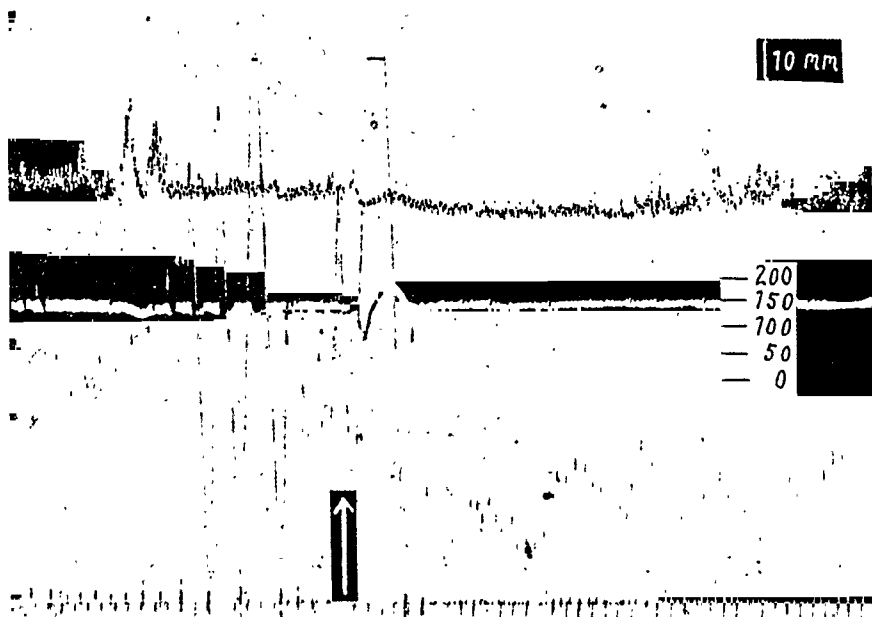


Fig. 2. Cat, 3.5 kg, chloralose. From above: Motility of the gut, blood pressure, rate of gastric secretion, time in minutes. At the arrow injection of 10 mg deseryl/kg.

In four animals we investigated the effect of deseryl without histamine. There were altogether six injections of the drug. In all these experiments there was a "spontaneous" secretion of low rate which was in no case reduced by deseryl. On the contrary deseryl notably increased the secretory rate in three cases. Fig. 2 shows the one experiment in which the increase was most marked.

As the antihistamine action of β -dimethylaminoethyl benzhydryl ether hydrochloride has been studied especially on smooth muscle we registered the motility of the intestine as a control. From these experiments it is obvious (fig. 1) that deseryl strongly antagonises the action of histamine on the gut. The effect appears almost immediately after the injection. In four of six experiments the motility of the gut was completely inhibited throughout the observation time (up to 5 hours). In two experiments a reduced motility reappeared after 15–30 minutes and when injection of deseryl was repeated the motility again ceased completely. In those cases where histamine was not injected the spontaneous motility of the gut was not so markedly diminished by deseryl.

In the doses employed in our experiments deseryl caused a

fall in blood pressure of short duration, especially after rapid injection, followed by a rise in blood pressure. After a few minutes the blood pressure had reached the original level. Micturition during the injection was found to occur regularly. An increase in sensitivity to external stimuli also seems to follow the injection of deseryl.

Discussion.

In our experiments we have thus not observed any antagonism between deseryl and histamine on gastric secretion in the cat. The very short reduction of secretion observed in some experiments has appeared synchronously with the fall in blood pressure and might be caused by it. Another possible explanation may be that deseryl suddenly diminishes the motility of the stomach thus interfering with the outflow of juice. The augmentation in secretion rate can not be due to the rise in blood pressure as this is of very short duration. We have not the impression that deseryl has any effect on the viscosity of the gastric juice which could explain the alteration in drop rate. We believe that the increase in secretion after deseryl is due to the fact that deseryl itself slightly stimulates gastric secretion. It may be added that in 1 experiment of 18 McELIN and HORRAN (1946) found that benadryl seemed to provoke a rise in gastric acidity in man.

It is well known that β -dimethylaminoethyl benzhydryl ether hydrochloride specifically antagonises histamine on isolated guinea-pig gut. From our experiments it is obvious that deseryl has the same effect on cat's intestine in situ. We have not investigated whether the antagonism is specific to histamine but it may be mentioned that the spontaneous motility does not seem to be markedly reduced by deseryl. It is thus clear that the dose of deseryl used in our experiments was sufficient to antagonise histamine on one of its effects. The dose used was high and when we doubled or trebled it the cat died.

It is noteworthy that two substances, thymoxiethyldiethylamine and β -dimethylaminoethyl benzhydryl ether hydrochloride, with such marked antihistamine action on smooth muscle do not antagonise histamine-induced gastric secretion. LOEW and CHICKERING (1941), who found that thymoxiethyldiethylamine does not antagonise histamine on gastric secretion concluded that the

mechanism by which histamine releases contraction of smooth muscle is of another kind than that by which secretion of gastric juice is elicited. Our experiments seem to support this view.

Summary.

β -dimethylaminoethyl benzhydryl ether hydrochloride in a dose which strongly antagonises the effect of histamine on the motility of the gut has no antihistamine action on gastric secretion in cats under chloralose. On the contrary there is an increase in the rate of secretion. This may possibly be due to the fact that the drug itself slightly stimulates gastric secretion.

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The Relative Importance of Ultrafiltration and Secretion in the Formation of Aqueous Humour as revealed by the Influence of Arterial Blood Pressure on the Osmotic Pressure of the Aqueous.

By

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Introduction.

In a previous paper (BÁRÁNY 1947) I have demonstrated that sodium enters the aqueous predominantly by secretion. This implies either

- (1) that practically no ultrafiltration at all takes place from the vessels of the iris and ciliary body or
- (2) that the amount of sodium entering the aqueous by secretion greatly exceeds the amount entering by ultrafiltration, even if ultrafiltration still supplies a considerable part of the fluid volume of the aqueous.

If alternative (1) were true, the old question of the relative importance of ultrafiltration and secretion in aqueous formation would be settled. If alternative (2) were true, the problem would still be unsolved. The present paper, therefore, is concerned with the question of whether alternative (2) is true.

If the secretion is to supply almost all the sodium, and the ultrafiltrate a considerable part of all the fluid, their respective sodium-contents obviously must be markedly different. But as sodium is the absolutely dominant cation of the aqueous, a marked difference in osmotic pressure would necessarily accompany any large difference in sodium-content. If alternative (2) were true, therefore, the secretion would have a higher osmotic pressure than the ultrafiltrate. The aqueous, being a mixture

of the two, would have an intermediate osmotic pressure, and this would depend on the proportions of the mixture. Thus, by reducing the amount of ultrafiltrate, one would change the osmotic pressure of the aqueous towards that of the secretion, and, as the secretion is hypertonic, towards higher values of osmotic pressure. This consequence of alternative (2) is tested in the present paper.

The amount of ultrafiltrate (if any) in the aqueous of one eye was reduced by clamping of the homolateral common carotid artery. It has been shown previously that this would greatly reduce the filtering pressure and thereby the rate of ultrafiltration (*loc. cit.*). Consequently, if alternative (2) were true, some time after carotid closure the amount of ultrafiltrate in the aqueous would be reduced and the osmotic pressure increased above that of the control eye with normal blood pressure.

Methods.

Rabbits with a van Leersum carotid loop were used. The arterial blood pressure of one eye was reduced by clamping the loop some 1.5—2 hours before aqueous samples were taken from both eyes. This time is sufficient for most of the aqueous to be renewed. The blood pressure was estimated by means of an ear capsule as previously described. (*loc. cit.*) Shortly before the aqueous was withdrawn the animals were anaesthetized with 0.6 ml Numal "Roche" (allyl-isopropyl-barbituric acid) intravenously, diluted with a few ml normal saline.

Aqueous was drawn from both eyes exactly as previously described. The amounts actually obtained varied somewhat, depending on the amount of air leaking into the syringe. In no case was more than 0.25 ml taken but sometimes much less. In order to avoid differences of concentration within the sample, a small amount of air was drawn into the syringe in those cases where no air had entered accidentally and the contents of the syringe were stirred by means of the air bubble. Special control experiments showed that no appreciable evaporation error was thus introduced. The samples were kept in the syringes with needles *in situ*, in the humidified box on top of the water bath (see below). Because the same needle was used for puncturing the cornea and for placing the aqueous on the thermopile for vapour pressure determination, it was important to prevent small amounts of aqueous from drying on the outside of the needle tip. This was therefore very carefully dried with filter paper after the anterior chamber puncture. Initial neglect of this precaution spoiled many experiments.

In most cases each eye was punctured at least twice. Sufficient time was always allowed for the eye to recover completely and each eye was examined in the slit-lamp microscope and macroscopically before

use. Irritation was extremely rare and such eyes were discarded, as were eyes with aqueous flare but lacking other signs of irritation.

The osmotic pressure difference between the two aqueous humours was determined by means of Hill-Baldes' thermoelectric vapour pressure method. A description of the slightly modified element and pile construction used will be given elsewhere. As recommended by BALDES and JOHNSON (1939), a large humid chamber accommodating three piles at once was used and the water bath was topped by a humidified box for the loading of the piles and the keeping of the syringes.

The aqueous samples usually were sufficiently large to allow some 6 to 9 independent determinations of osmotic pressure difference but sometimes there was enough only for 3. Sometimes a value would fall very much outside the rest of the group. These values were not discarded but by using the medians of the groups as statistical units rather than the means the influence of abnormal errors was minimised.

The sensitivity of the arrangement was about 3.51 mm scale-deflection per 1 mM NaCl, with insignificant variations between different piles. The calibration curve was a straight line through the origin. The standard error of a single determination of osmotic pressure difference (method error) was less than 0.43 mM NaCl, using 0.9 per cent against 0.89 per cent NaCl (53 determinations) and, using the same solution on all thermojunctions, 0.28 mM NaCl (73 determinations). The method error, using aqueous humour, was 0.37 mM NaCl (100 determinations picked at random). Thus, the standard error of the method was of the order of magnitude of 0.2 per cent of the total concentration, which is in complete accordance with the figures given by Baldes on various occasions.

Results.

The blood-pressure reduction caused by carotid clamping was estimated in 28 of the 29 experiments done and accidentally omitted in one. The average mean blood pressure obtained from the ear capsule readings was 42.0 mm Hg on the clamped side and 60.8 on the control side. The mean ratio was 0.705. This corresponds to a reduction in mean ophthalmic artery pressure of 30 mm Hg, and thus would have caused a very substantial reduction in filtering pressure if ultrafiltration actually occurred (Cp. BARÁNY 1947).

Table I shows how the blood-pressure drop influenced the osmotic pressure of the aqueous. The table summarizes the results of 29 experiments with reduced blood pressure (on 22 animals) and 23 control experiments with open carotid loop (on 20 animals, largely the same). The relevant columns are those showing the medians, the means are given only for comparison.

It is seen at once that *no significant osmotic pressure difference*

Table I.

Animal n:r	Date	Number of determinations	Osmotic pressure difference in mM NaCl Control side — Side with carotid loop			
			Carotid occluded		Carotid open	
			Mean	Median	Mean	Median
501	7-12-45	6	-1.3	-1.4		
»	3-1-46	4			+1.9	+2.0
»	24-1	6	+1.3	+1.1		
563	19-1	2			+1.3	+1.2
592	21-12	6	+0.3	+0.2		
»	9-1	6			-0.3	-0.3
»	22-1	9	+1.1	+1.2		
»	19-2	6	+0.1	+0.1		
593	20-12	6	-0.5	-0.5		
»	4-1	6			-0.1	+0.3
»	14-2	6	-0.8	-0.9		
599	12-2	6	+0.5	+0.7		
602	19-12	5	-2.4	-2.1		
603	5-12	6	+0.3	+0.2		
604	7-1	9			-0.3	-0.3
»	16-2	6	+0.3	+0.4		
606	6-12	9	+0.1	-0.3		
608	8-1	6			+1.0	+0.8
609	13-12	6	-3.3	-3.2		
610	6-12	3	-0.2	± 0.0		
»	15-12	9	+0.3	+1.0		
»	20-3	6			-0.4	-0.4
613	20-2	6	+0.1	+0.2		
»	9-3	6			-0.5	-0.6
628	9-2	4	+0.6	+0.5		
»	25-2	4	-0.3	-0.2		
»	19-3	6			+0.3	+0.3
631	11-2	3	-2.0	-1.7		
»	28-2	3	+1.1	+1.1		
»	18-3	9			+0.1	+0.7
635	23-1	6			-0.7	-0.9
»	7-2	6	-0.3	-0.1		
636	18-2	6	+1.4	+1.4		
»	16-3	6			-1.0	-0.9
639	31-1	6			-0.3	-0.1
»	13-2	4	+0.2	+0.1		
»	7-3	5			+0.5	+0.5
640	29-1	6			+0.5	+0.3
»	8-2	6	-0.4	-0.4		
»	11-3	6			-0.3	-0.3
641	15-2	6	-0.4	-0.3		
»	26-2	6			-1.0	-1.0
642	6-2	6			-2.2	-2.0
643	22-2	3	-0.1	-0.2		
»	21-3	6			-0.2	-0.2
645	26-1	6			-1.0	-0.5
»	21-2	6	-0.6	-0.5		
646	25-1	6			+0.5	+0.5
»	15-3	6	-0.2	-0.1		
648	28-1	9			-0.1	± 0.0
»	23-2	4	-0.2	-0.2		
»	8-3	6			-0.3	-0.2
Mean			-0.13	+0.08± ± 0.19 σ = 1.02	-0.11	-0.05 ± 0.17 σ = 0.82

exists between the aqueous of the side with clamped carotid and that of the control side. If closing the carotid increases the osmotic pressure of the aqueous, the increase is certainly less than $3 \times 0.19 - 0.08 = 0.49$ mM or $3.2/1000$ of the total osmotic pressure, if the osmotic pressure of the aqueous is taken as equal to 0.9 per cent or 154 mM NaCl. This is, of course, a very small change in concentration, and the result is therefore quite incompatible with alternative (2) of the introduction, namely that ultrafiltration is responsible for a considerable part of the volume of the aqueous.

Thus, the experiments show that ultrafiltration is of small or no importance in the formation of aqueous humour.

Besides this main result, the other figures are of smaller interest. It may be noted, however, that the standard error of the determinations of osmotic pressure difference between the eyes is about 0.9 mM NaCl (1.02 resp. 0.82). This standard error represents the scatter of the medians of 3—9 single measurements. Since the method error of one single osmotic measurement is only at most 0.43 mM NaCl, the method error of the osmotic measurement contributes only insignificantly to the scatter of the values. Thus the accuracy of the thermo-electric method is more than sufficient for aqueous humor studies and the scatter of the values is mainly an expression of actual differences between the two aqueous samples, as present in the syringes. Whether the differences existed already before the samples were withdrawn, it is impossible to say.

Finally it is of some interest to consider how large an osmotic pressure difference between the aqueous humours would be required in order to be detectable by the present method. This, of course, depends on how many experiments are made. The standard error of 0.9 mM NaCl corresponds to 30—35 mm Hg osmotic pressure. In order definitely to prove the existence of a difference of 10 mm Hg, one would thus need about 100 experiments! This is, as pointed out above, not the fault of the thermo-electric method but possibly of the aqueous sampling method or of the inherent variability between the eyes.

Discussion.

The result arrived at is clearcut enough: ultrafiltration cannot play any important rôle in the formation of aqueous humour. This, of course, is what has been held by the proponents of the

secretory theory of aqueous formation for many years. The most important argument in favour of their theory was that the vascular pressures would not be sufficient to allow ultrafiltration to take place. The measurements of SEIDEL (1924) showed the arterial pressure in the vessels of the iris and ciliary body of man to be diastolic 30—45 mm Hg and systolic 55—75 mm Hg. Thus, the mean arterial pressure 42—60 mm Hg would be barely sufficient to ensure ultrafiltration — the sum of intraocular pressure and colloid-osmotic pressure of the plasma being about 50 mm Hg. Assuming the pressure drop in the arterioles to be of the same order of magnitude as elsewhere in the body, the figures would in fact exclude the possibility of ultrafiltration. Nothing is known, however, about the actual pressure drop in the arterioles in question and therefore the measurements of SEIDEL have not generally been considered to settle the question. They fit in very well with the present findings, however.

Summary.

Sodium has been shown to enter the aqueous predominantly or wholly by secretion. Therefore, if the aqueous were a mixture of a secretion and any considerable amount of ultrafiltrate, the osmotic pressures of these two components would have to be markedly different. A blood pressure reduction which changes the amount of ultrafiltrate in the aqueous would consequently change the osmotic pressure of the aqueous.

The osmotic pressure difference between the aqueous humours of both eyes was measured in rabbits after the blood pressure of one eye had been kept at 30 mm Hg below the normal level for 1.5—2 hours. No significant difference in osmotic pressure was detected. Therefore, the amount of ultrafiltrate in the aqueous cannot be considerable.

This investigation has been made possible by a grant from the Lennander Foundation of the University of Uppsala. Miss G. THELIN and Mr. A. RÜÜTLI have given most valuable assistance.

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A Specific Micro-method for Colorimetric Determination of Methanol in Blood.

By

KJELL AGNER and KARL ERIK BELFRAGE.

Received 10 October 1946.

EEGRIFE (1937) reported the use of chromotropic acid (1,8-dihydronaphthalene-3,6-disulphonic acid) as a specific reagent for formaldehyde. After the addition of formaldehyde the reagent in 72 % sulphuric acid developed a purple colour when heated to 60°. Alcohols, ketones, carboxylic acids, and other aldehydes are among the substances tested which do not react. MACFAYDEN (1945) and BRICKER and JOHNSON (1945) have independently investigated the conditions which affect the development of the purple colour. They described experimental conditions under which the extinction, measured in a Beckman quartz prism spectrophotometer, was proportional to the formaldehyde concentration.

The chromotropic acid reaction has also been used for determination of substances which can be converted to formaldehyde. BOYD and LOGAN (1942) oxidized serine to formaldehyde with sodium periodate, and MACFAYDEN (1945) and ALEXANDER, LANDWEHR and SELIGMAN (1945) combined the ninhydrin and the chromotropic acid reaction to provide a specific method for glycine. MACFAYDEN (1945) stated that he was investigating the possibility of oxidizing methanol to formaldehyde with a view to a quantitative estimation of methanol by his procedure. By the time MACFAYDEN's article reached us we had almost concluded the investigations (1946) reported here. Our investigations have been directed towards working out a micro-method for the determination of methanol in blood.

Experimental.

The most suitable conditions for the reaction of formaldehyde with chromotropic acid have been investigated. The results were, on the whole, in agreement with those earlier described by MACFAYDEN and BRICKER and JOHNSON.

Various methods for the oxidation of the methanol have been investigated: molecular oxygen and finely divided platinum, potassium permanganate in acid and alkaline solution, potassium

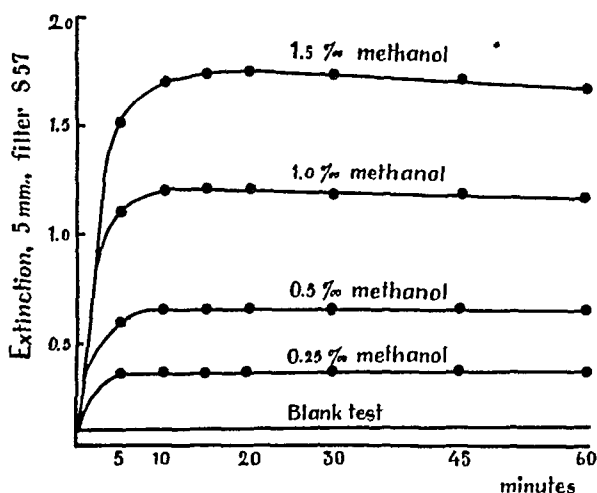


Fig. 1. Relationship between the extinction, 5 mm., filter S 57, Pulfrich Photometer, and the time of oxidation for different methanol concentrations. To 0.1 ml. methanol was added 0.1 ml. permanganate solution. The excess of permanganate was reduced by sulphur dioxide after different times, 20 mg. chromotropic acid in 4 ml. sulphuric acid was added. Heating at 100° C in 30 min.

bichromate, sodium persulphate, magnesium peroxide in sulphuric acid etc. On oxidation of the methanol, other oxidation products besides formaldehyde may be formed. The experimental conditions have to be adjusted so as to give a maximum amount of formaldehyde and a minimum of the other products. The formaldehyde formed should not be oxidized further, or in any case its rate of oxidation must be very low. Among the oxidation reagents investigated, a saturated solution of permanganate in phosphoric acid has proved to be the most suitable. (Fig. 1.) The oxidation was carried out at room temperature. The excess permanganate was destroyed by sulphur dioxide, which was conducted into the tube in which the oxidation was performed.

The value for the extinction was approximately constant over oxidation periods of 15—30 min., and was proportional to the original methanol concentration. For determination of the methanol concentration in blood this oxidation method has been combined with the precipitation of protein with trichloroacetic acid.

Method.

Reagents:

Heparin solution: 0.25 ml of 5 % heparin in 100 ml of water.

Trichloroacetic acid: 25 %.

Potassium permanganate solution: 50 ml of diluted phosphoric acid (2.5 ml of chemically pure phosphoric acid, density 1.75, diluted to 50 ml) is saturated with potassium permanganate, about 2.25 gr.

Sulphur dioxide: from a tube containing sodium pyrosulphite and sulphuric acid, sulphur dioxide is led off into the tube in which the oxidation has been carried out.

Chromotropic acid¹ solution: for each test 20 mg chromotropic acid in 4 ml of 72 % sulphuric acid (100 ml of distilled water + 150 ml of concentrated sulphuric acid) was used.

Procedure:

Two-tenth ml of capillary blood was hemolyzed in a centrifuge tube containing 0.20 ml of heparin solution. — If one expects a higher methanol concentration than 3 ‰, one should measure out 0.1 ml of blood and 0.3 ml of heparin solution. — After complete hemolysis 0.2 ml of 25 % trichloroacetic acid was added, and the solution centrifuged in a closed tube. One-tenth ml of filtrate was pipetted into a test tube and oxidized with 0.1 ml of permanganate solution for 20 min. Then the excess of permanganate was reduced with sulphur dioxide. 4 ml of a mixture of chromotropic acid and sulphuric acid was added, followed by heating in boiling water bath for 30 min. The test tubes were closed with rubber stoppers. After cooling, the value for the extinction was read with a Zeiss' Pulfrich Photometer.

For the determination of the extinction developed from blood with a known methanol concentration, 99.5 ml of normal blood was mixed with 0.5 ml of methanol (the alcohol concentration being 4 ‰). This blood was diluted with normal blood in order to obtain samples with a lower methanol content. The different samples were treated in the way described above, and the extinction was read off by using colour filters S 50, S 53, S 57, and S 61, transmitting at 500, 530, 570, and 610 m μ respectively fig. 2. The methanol contents of the different blood samples were also determined with Widmark's micro method as adapted for the determination of methanol by BILDSTEN (1924), and subsequently somewhat modified by LINDE² (1932). Table 1.

¹ Mercks Chromotropsäure für Tüpfelanalyse nach Feigl and Chromotropic acid from the Eastman Kodak Company have been used.

² The analyses have been carried out by Docent L. GOLDBERG, to whom the authors are indebted for his kindness in placing his experience and his trained staff at their disposal.

Table 1.

Calculated methanol concentration	Methanol concentration determined according to Widmark
0.50	0.51
1.00	1.05
1.50	1.56
2.00	2.08
2.50	2.49

From fig. 2 it is seen that for normal blood the extinction $E_{S\ 50} > E_{S\ 53} > E_{S\ 57} > E_{S\ 61}$. With a methanol concentration of about 0.25 ‰ $E_{S\ 57} = E_{S\ 50} > E_{S\ 53}$, and at higher alcohol concentration a distinct maximum for the extinction is found at S 57. The extinctions for the different samples were proportional to the methanol concentration.

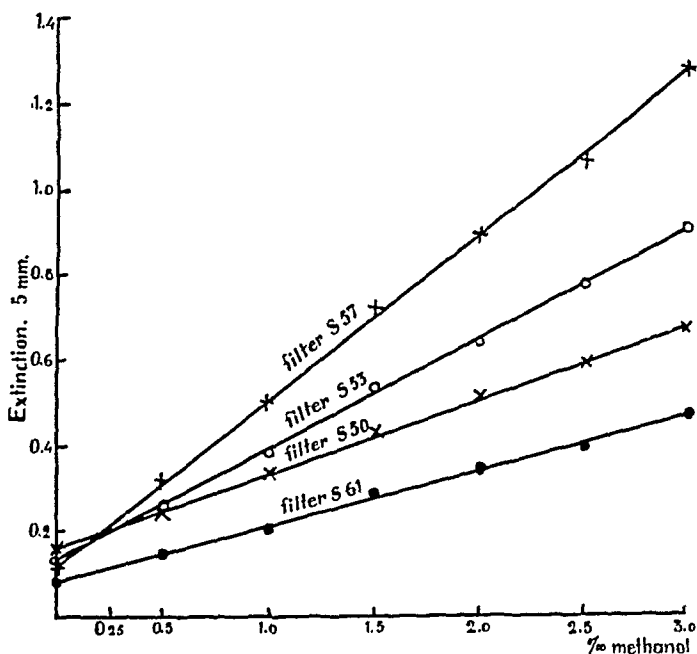


Fig. 2. Relationship between extinction and the concentration of methanol in blood.

For the calculation of the methanol concentration (mg methanol per ml blood) we have used the value for the difference $E_{S\ 57} - E_{S\ 61}$ for the sample, reduced by the corresponding value for normal blood, and set up the following formula:

$$\text{‰ methanol} = k \times \left[\frac{(E_{S\ 57}^{5\text{ mm}} - E_{S\ 61}^{5\text{ mm}})_{\text{test}}}{(E_{S\ 57}^{5\text{ mm}} - E_{S\ 61}^{5\text{ mm}})_{\text{normal blood}}} \right]$$

The value for the proportionality factor k was 4.25 with the colour filters used by us.

The difference $E_{S\ 57}^{5\text{ mm}} - E_{S\ 61}^{5\text{ mm}}$ has been determined for about one hundred samples of normal blood and has varied within the limits 0.040—0.050.

For the checking of low values the extinction is also read off with filter $E_{S\ 50}$. Methanol concentration: $> 0.25\text{ ‰} : E_{S\ 57} > E_{S\ 50}$
 $< 0.25\text{ ‰} : E_{S\ 50} > E_{S\ 57}$

The elimination of methanol.

The method described above has been used for determining the elimination of the methanol. Investigations have also been made to ascertain whether formaldehyde can be demonstrated in blood in cases of methanol intoxication, and whether the presence of ethanol affects the elimination of the methanol.

With Widmark's method (1922), adapted for the determination of methanol by BILDSTEN, BILDSTEN (1924), BILSDTEN and WIDMARK (1924) as well as BERNHARD and GOLDBERG (1933) have determined the rate of fall of the concentration of methanol in the blood, arriving at the approximate figure 0.0007 ‰ per min. Four to six hours after the injection the concentration curve showed a temporary maximum.

Methanol was given to rabbits intravenously and orally with a rubber tube. The methanol content was determined with the method described above after the lapse of different periods of time. (Fig. 3.)

During the first hours of the experiments, when the methanol concentration was still high, the concentration decreased relatively rapidly, or about 0.0017 ‰ per min. During the later part of the experiments, however, the rate of elimination was reduced to about 0.0006 ‰ per min. In our experiments we did not find the maximum in the concentration curve shown by BILDSTEN (1924). BILDSTEN asserts that this rise in the concentration curve was probably caused by volatile, reducing substances formed on the oxidation of the methanol in vivo, and which caused too high values on determination with the bichromate method. These substances do not, however, react with chromotropic acid.

BRÜCKNER (1924), FLURY and WIRTH (1936), and other investigators have assumed that formaldehyde was formed as an intermediate product when methanol was oxidized in the organism. KEESER (1931) demonstrated formaldehyde in the cerebro-spinal

fluid, the vitreous humour of the eye, etc. in methanol intoxication and together with VINCKE (1940) he found that the liver tissue oxidized methanol to formaldehyde.

With the chromotropic acid reaction we have carried out tests to ascertain if formaldehyde occurred in the blood of the intoxi-

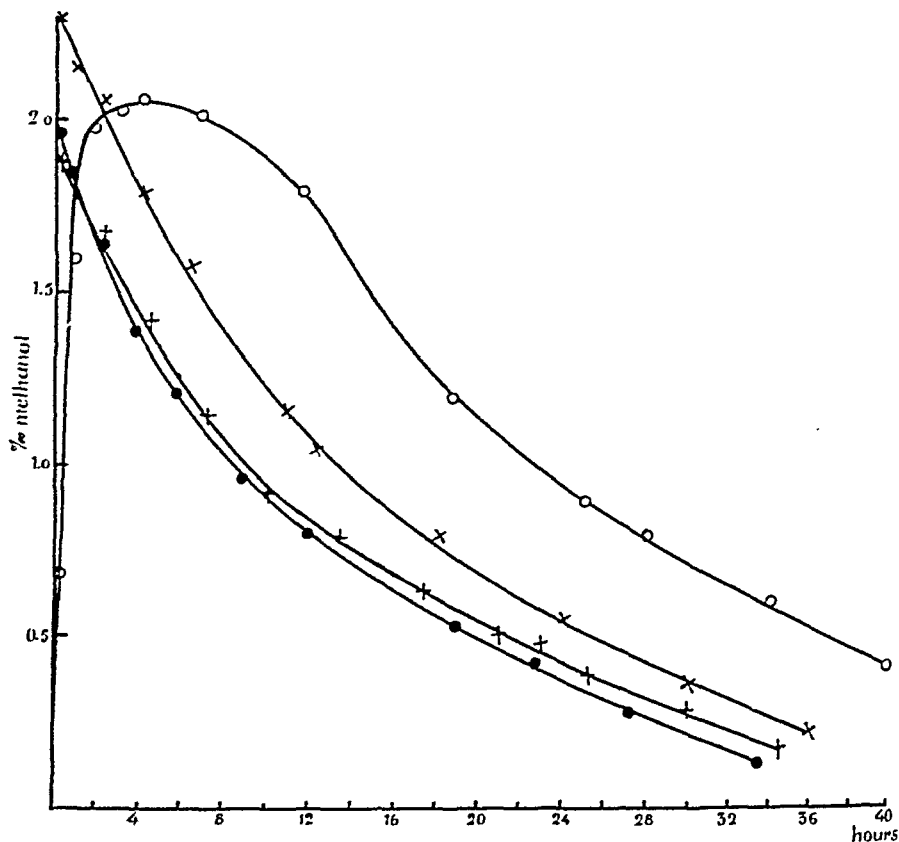


Fig. 3. mg. methanol pro ml. blood.

1. ×—×—× 1.9 gr methanol pro kg., intravenously injected.
2. ●—●—● 1.7 " " " " " "
3. +—+—+ 1.7 " " " " " "
4. ○—○—○ 2.2 " " " " orally "

cated rabbits. It was not possible, however, to demonstrate formaldehyde at any time. The volatile substances that caused a temporary rise of the concentration curve in BILDSTEN's and in BERNHARD and GOLDBERG's experiments are thus of a nature other than formaldehyde.

ASSER (1914) observed that methanol intoxication often caused only slight symptoms of poisoning in persons who were "notorious schnaps drinkers". He also investigated the formate secretion in

urine from animals that had been given, besides methanol, also ethyl alcohol, amyl alcohol or acetone. The secretion of formate, which was considerably increased in cases of methanol intoxication, was in these cases only slightly increased. He drew the conclusion that the mode of oxidation of the methanol is changed when other alcohols are also present. RØE (1943) has communicated clinical observations resembling those reported by ASSER.

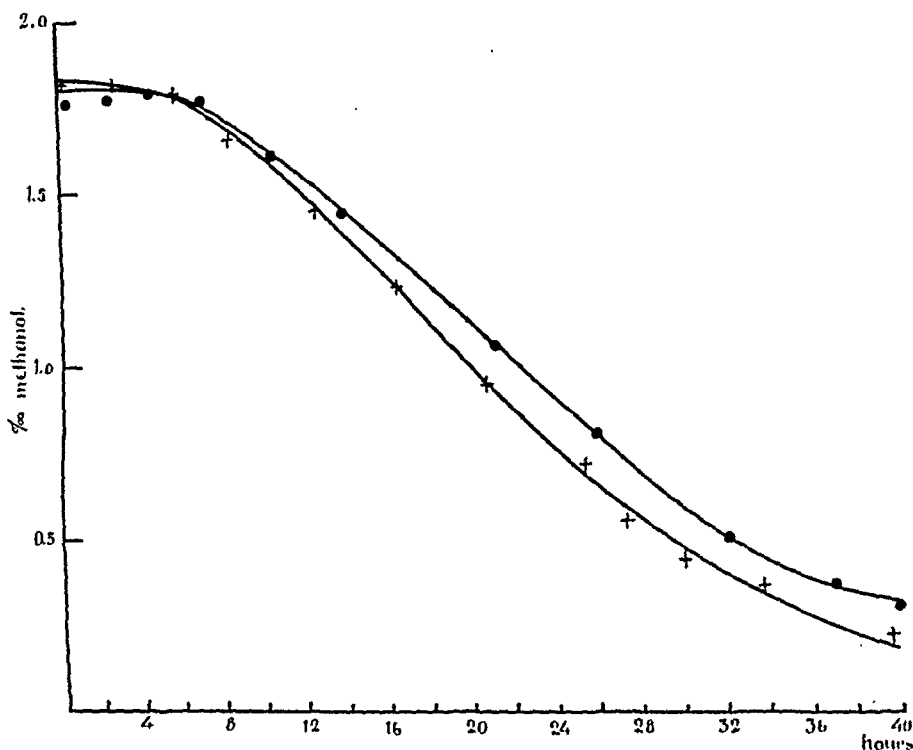


Fig. 4. mg. methanol pro ml. blood.

5. ●—●—● 1.7 gr methanol + 1.7 gr ethanol pro kg., intravenously injected.
 6. +—+—+ 1.7 " " " " " " " " " " " "

The above-described method of determination is specific for methanol, and it has therefore been possible to use it to determine whether administration of ethanol simultaneously with methanol has any effect on the elimination of the methanol. Ethanol and methanol were simultaneously injected into rabbits, after which the methanol concentration was determined after the lapse of different periods of time. (Fig. 4.) Rabbits of the same weight and age as in the previous experiment were chosen. In these experiments the amounts of methanol were as great as for rabbits 2 and 3.

During the first 6—7 hours of the experiments the methanol concentration was practically constant. After this period the elimination curves showed approximately the same appearance as in fig. 3. The oxidation of methanol in the organism was evidently prevented by the simultaneous occurrence of ethanol.

Summary.

1. A method for colorimetric determination for methanol has been described.

2. The rate of elimination of methanol in blood has been determined. At a methanol concentration of more than about 1 ‰ the rate of elimination was about 0.0017 ‰ per min. and at lower concentrations the value was about 0.0006 ‰ per min.

3. It has not been possible to demonstrate formaldehyde in blood in cases of methanol intoxication.

4. The simultaneous occurrence of ethanol prevents the elimination of methanol.

The authors' thanks are due to Stiftelsen Therese och Johan Anderssons Minne for grants.

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From the Physiological Institute, University of Uppsala.

The Action of Atropine, Homatropine, Eserine and Prostigmine on the Osmotic Pressure of the Aqueous Humour.

By

ERNST H. BÁRÁNY.

Received 18 October 1946.

Among the factors which govern the intra-ocular pressure, the osmotic relations between blood and aqueous humour play an important and well-recognized part. I have therefore studied the influence on the osmotic pressure of the aqueous of some drugs which — at least under certain conditions — may be able to influence the intra-ocular pressure. While the action of the mydriatics and miotics on the pupil, on the vascular reactions of the eye and on the permeability of the blood-aqueous barrier have been fairly intensely studied, nothing seems to be known about their influence on the osmotic pressure of the aqueous humour.

Methods.

The osmotic pressure difference between aqueous samples obtained from the experimental eye and the control eye of rabbits was determined by means of HILL-BALDES' thermo-electric osmometer. Data on the method of collecting aqueous and on the sensitivity and method error of the arrangement used have been given elsewhere (BÁRÁNY 1947 a, b). From 3 to 6 determinations of osmotic pressure difference were made on each pair of aqueous samples and the medians of the values obtained were used as the statistical unit.

Rabbits of 2—3 kgm weight, mostly male albinos, were used. The drugs were given in aqueous solution, the standard dose being 2 drops from a standard dropper into the lower conjunctival sac. The following concentrations were used: atropine sulfate 1.0 and 5.0 %, homatropine

hydrobromide 1.0 %, eserine salicylate 0.5 % and prostigmine bromide 3 %. The two latter drugs were tested both on eyes which had never received them before and on eyes which for some time had been habituated to the drugs. During this habituating period, the animal received 2 drops once or usually twice daily. Several of the atropine animals had previously received an occasional drop of atropine but were not systematically habituated to the drug.

Conjunctival instillation obviously is not an exact method of drug dosage. In the present connection, however, the object was only to obtain a strong effect and there was no need for an exact dosage.

Eserine and prostigmine were given at varying intervals before the aqueous was withdrawn. These intervals are listed in the respective tables. There was always an intense miosis. Atropine and homatropine were instilled repeatedly with 20 minutes intervals, the last instillation about 20 minutes before the aqueous samples were taken. The number of instillations is listed in the table.

Results.

The results from the mydriatic group are summarized in Table I. The first 4 experiments were done with 1 per cent atropine. As

Table I.

Drug	Animal n:r	Date	Number of instilla- tions	Number of det:ns	Osmotic pressure difference in mM NaCl Control side — experimental side.
Atropine 1 %..	650	1/2 46	3	6	+ 0.46
	652	2/2 »	3	6	+ 0.51
	654	4/2 »	3	6	+ 0.38
	653	5/2 »	3	6	— 0.46
					Mean + 0.18
Atropine 5 %..	593	22/3 »	3	4	+ 0.51
	636	26/3 »	2	6	— 0.91
	592	27/3 »	2	3	+ 0.31
	645	28/3 »	2	6	+ 0.73
	641	29/3 »	2	6	+ 0.09
	613	30/3 »	2	6	+ 0.68
	631	1/4 »	2	6	— 0.17
					Mean + 0.18
Homatropine 1 %	640	3/4 »	3	3	— 0.54
	646	9/4 »	3	6	+ 0.23
	610	10/4 »	3	4	+ 0.48
	593	11/4 »	3	6	+ 0.40
	642	12/4 »	3	6	+ 0.58
	645	13/4 »	3	6	+ 1.06
					Mean + 0.37
Total mean					+ 0.24 ± 0.12

the resulting osmotic pressure differences were only of the order of magnitude of the standard error it was decided to try a stronger solution. The 7 experiments with 5 per cent atropine were consequently made. Only one drop was given each time, however. All the same, a very marked mydriasis of the contralateral eye was observed obviously resulting from absorption of the drug into the general circulation. As drug action on the control eye might possibly mask the drug effect on the experimental eye, a further 6 experiments were made with 1 per cent homatropine, the action of which is so weak that no influence on the opposite eye has to be feared. The homatropine dose was sufficient, however, to give a maximal mydriasis on the treated side.

The table shows that atropine and homatropine possibly tend to lower the osmotic pressure of the aqueous. The effect observed is small, however, only about 10 mm Hg, and is merely twice its standard error, even if all experiments are made into one group. It might, therefore, be due to random variation. On the other hand, its smallness may depend on systemic absorption and action on the control eye in the atropine experiments. The higher values obtained in the homatropine experiments point in this direction.

The action of eserine on eyes which had previously never received the drug is shown in Table II. Aqueous was withdrawn at a time when — according to LARSSON (1930) — the initial pressure increase and iris hyperaemia have subsided and the intra-ocular pressure is reduced. In this hypotonic phase there is always an increased protein content of the aqueous, which was also seen in our animals as an aqueous flare on slit-lamp examination.

The table shows that eserine in previously untreated eyes seems to lower the osmotic concentration of the aqueous humour. The value obtained, 0.74 ± 0.31 mM NaCl is about 2.5 times its standard error. It is thus statistically probable. The corresponding reduction in osmotic pressure is 25—30 mm Hg.

This effect of eserine on previously untreated eyes seems to differ from that observed in eyes which were habituated to the drug, so that they did not show any aqueous flare after instillation. Table III shows that the drugs had practically no influence on the osmotic pressure of the aqueous in these tolerant eyes. This is true both of eserine and prostigmine. In view of the close similarity of action between the drugs, the experiments from the

Table II.

Animal n:r	Color	Date	Interval between drug instillation and withdrawal of aqueous, minutes	Number of det:ns	Osm. pressure difference, mM NaCl Control side — experimental side
1	not anno- tated	6/6 46	115	3	— 0.11
2	"	7/6 "	133	6	+ 2.82
14	albino	8/6 "	106	4	+ 3.09
10	"	11/6 "	117	5	— 0.17
958	black	12/6 "	91	3	+ 3.00
961	albino	12/6 "	115	5	— 1.25
935	"	13/6 "	85	6	+ 0.78
B 16	"	29/6 "	180	6	+ 0.74
980	"	1/7 "	135	5	— 0.57
B 14	"	2/7 "	150	5	+ 0.84
3	grey	3/7 "	160	6	+ 2.35
B 28	albino	4/7 "	165	3	+ 1.25
B 21	"	5/7 "	not annotated	3	+ 1.59
B 41	"	6/7 "	160	6	+ 0.11
B 43	"	8/7 "	180	6	— 1.46
B 44	grey	9/7 "	163	6	— 0.24
4	"	10/7 "	180	6	+ 0.48
5	"	11/7 "	150	3	+ 1.05
6	albino	12/7 "	150	3	— 0.97
7	"	13/7 "	150	6	— 1.54
					Mean + 0.74 \pm 0.31

two groups have been pooled, but even then no significant effect is discernible.

That the result is so close to ± 0 is of course a coincidence. There might be a small effect hidden behind the standard error. Our experiments have not been able to demonstrate it, however.

As LARSSON (1930) has shown in rabbit experiments, the action of eserine on a previously untreated eye differs from that on an eye which has become tolerant to the drug. In this latter case, there is no initial phase of intense iris hyperaemia and intra-ocular pressure rise and no gross increase of capillary permeability, leading to an aqueous flare. The main action of eserine in such an eye is a moderate decrease of intra-ocular tension, lasting for several hours. We have found prostigmine in this respect exactly to resemble eserine. Moreover, habituation to eserine prevents prostigmine from eliciting a protein increase in the aqueous and vice versa.

Table III.

Drug	Animal no.	Color	Date	Period of habitua- tion, days	Interval between drug instillation and with- drawal of aqueous, minutes	Num- ber of det:ns	Osmotic pressure difference, mM NaCl — Control side — experi- mental side
Eserine	809	albino	18/7 46	30	135	4	+ 0.16
	645	"	17/7 "	31	135	6	+ 1.62
	636	"	18/7 "	32	130	6	+ 0.14
	646	"	19/7 "	33	145	6	- 1.32
	593	"	20/7 "	34	135	5	- 0.14
	592	"	22/7 "	11	130	6	- 0.17
	803	grey	23/7 "	12	134	6	+ 0.27
	648	albino	24/7 "	13	170	6	- 0.77
	152	"	25/7 "	14	130	6	- 0.47
							Mean - 0.07
Prostig- mine..	648	albino	15/4 46	5	60	4	- 1.86
	613	grey	23/4 "	12	70	6	- 0.20
	646	albino	24/4 "	13	75	6	+ 1.02
	631	grey	25/4 "	14	68	6	- 0.45
	610	albino	26/4 "	4	70	6	+ 0.65
	592	"	29/4 "	7	70	5	+ 0.40
	604	"	30/4 "	8	70	6	+ 0.68
							Mean + 0.03
Total mean							- 0.03 \pm 0.22

Discussion.

1. The mechanism.

The decrease in osmotic pressure of the aqueous observed in the atropine group and in the untreated eserine group could be caused by at least two mechanisms. In view of the fact, that the main part of the aqueous solids, namely the sodium chloride — and probably the rest of the electrolytes — enter the aqueous by secretion (KINSEY and GRANT 1942, BÁRÁNY 1946, 1947 a) a reduced concentration in the aqueous might conceivably result either from a decrease in rate of secretion or an increase in rate of leakage of electrolytes out of the eye.

In the case of eserine it seems probable that the osmotic pressure decrease depends on an increased leakage of electrolytes out of the anterior chamber. The fact that electrolytes do not enter the aqueous by diffusion in the intact eye (KINSEY and GRANT 1942) shows that the normal blood-aqueous barrier must be fairly impermeable to electrolytes. When this relative imper-

meability is destroyed to such a degree that even protein will pass the barrier an interchange by diffusion between blood and aqueous certainly must occur. The observed fact that this interchange leads to a reduction in osmotic pressure of the aqueous is quite in keeping with the findings of several authors, that the normal aqueous is slightly hypertonic with respect to the blood. (HODGSON 1938, ROEPKE and HETHERINGTON 1940.) If the reduction in osmotic pressure by eserine were dependent on the breakdown of the normal blood-aqueous barrier, one would expect a different action of eserine in eyes habituated to the drug, where no such breakdown occurs. This is exactly what has been found.

In the case of atropine, -- if the effect observed is real and not due to random variation -- the same explanation might be true. The views concerning the influence of atropine on the blood-aqueous barrier are conflicting, but there are several authors who have observed a slight increase in permeability after atropine (LARSSON 1930, COLLE, DUKE-ELDER and DUKE-ELDER 1931). Such an increase would be expected from the dilatating action of the drug on the capillaries of the iris.

It is interesting to note that SWAN and HART (1940) in their study of the blood-aqueous barrier found a slight decrease in the total solids of the aqueous after atropine. This might be the same effect as was observed in the present investigation in the form of reduced osmotic pressure. The increase in non protein solids reported by the authors after eserine does not, however, agree with the present findings.

2. The significance.

It is probable that the reduction in osmotic pressure caused by eserine in previously untreated eyes is at least partly responsible for the hypotonia characterizing the late phase of eserine action in such eyes. Even if the vascular system is deranged in this phase and this derangement has an influence on the intra-ocular pressure (LARSSON 1930), a substantial reduction in osmotic water attraction to the aqueous cannot fail to influence the intra-ocular pressure.

The question lies near at hand, whether part of the anti-glaucomatous action of eserine could not be similarly explained. While the miotic action is certainly of highest importance in case of

obstructed filtering angle, there is an anti-glaucomatous effect of eserine even in cases where no obstruction of the angle can be detected. A reduction of the osmotic water attraction of the aqueous would furnish a basis for the anti-glaucomatous eserine effect in these cases.

This attractive possibility finds, however, no support in the present experiments.

In glaucoma, when the drug has been used for some time, eserine elicits no aqueous flare, no increase in aqueous protein. The action of the drug in glaucoma cannot, therefore, depend on any increase in *gross* permeability. Any important anti-glaucomatous action must be discernible also in an eye where eserine does not elicit a noticeable increase in aqueous protein. But in those of the present experiments where this condition was fulfilled, namely those on habituated eyes, no decrease in osmotic pressure was noted.

It must be remembered, however, that the present negative finding does not definitely settle the question. There might be something hidden behind the random variations which only further experiments can bring out.

Summary.

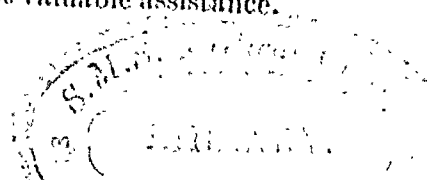
The influence of atropine, homatropine, eserine and prostigmine on the osmotic pressure of the aqueous humour has been studied in rabbits.

In previously untreated eyes eserine caused an osmotic pressure reduction equivalent to 0.74 ± 0.31 mM NaCl. In eyes habituated to eserine and prostigmine, no influence on the osmotic pressure was detected.

Atropine and homatropine caused a reduction in osmotic pressure equivalent to 0.24 ± 0.12 mM NaCl.

The decreases in osmotic pressure can probably be attributed to an increased permeability of the blood-aqueous barrier.

This investigation has been made possible by a grant from the Lennander Foundation of the University of Uppsala. Miss G. THELIN and Mr. A. RÜÜTLI have given most valuable assistance.



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From the Gynaecological Clinic of the Karolinska Sjukhuset.

A Quantitative Determination of the Content of Contractive Substances in Human Sperm and their Significance for the Motility and Vitality of the Spermatozoa.

By

JAN ASPLUND, Stockholm.

Received 19 October 1946.

About 1933—34, independently of each other, v. EULER and GOLDBLATT published fairly detailed investigations of a substance which could be proved in secretion from prostata and vesiculae seminales and in sperm from man, which v. EULER named "prostaglandin". This substance which is an organic acid soluble in lipoids is characterized chiefly by having a strongly lowering effect on blood pressure and a contractive effect on the smooth musculature. By comparing the characteristics of prostaglandin in respect of solubility in different media, its dialyzability, stability, biological effect, and the influence on it of atropine, v. EULER was able to show that prostaglandin could not be identical with certain previously known depressor substances, such as acetylcholine, adenosine, histamine, kallikrein and substance P. In his investigations GOLDBLATT bore in mind especially the possibility that the active substance might consist of acetylcholine, but considered that he could prove from the following three facts that such was not the case: Firstly, at the time of the investigations the specimens of sperm were generally 10 hours old, when any acetylcholine should have been destroyed, secondly, the contractions appeared after all too long periods of latency, and thirdly, atropine had no checking effect.

On the other hand, COCKRILL, KURZROK and MILLER, who in

an investigation in 1935 tested the effect of sperm on strips of human uterus and found that 65 out of 75 specimens of sperm had a relaxing effect on uterine musculature, while the other 10 always had a contractive effect, considered for several reasons that the substance consisted of acetylcholine.

v. EULER states that in the preparation of prostaglandin in pure form from sperm he obtained a precipitate soluble in ether, which consisted largely of choline. Other authors also, such as KAHANE and LEVY (1937) and FLETSCHER, BEST and SOLANDT (1935), state that sperm is extremely rich in choline. For the rest, the method which has long been known of proving small quantities of sperm for forensic medical purposes is based on the occurrence of choline in sperm.

Further, according to ZELLER and JOEL (1941), there are not inconsiderable amounts of histamine in sperm, which to a certain extent does speak in favour of the occurrence also of histaminase, although the author could not find any information on the point in the literature.

Thus it is probable that the capacity of the sperm to exert a contractive effect on smooth musculature is conditioned by several substances. Without attempting to analyse more closely the nature of these substances, but on the working hypothesis that they consist principally of prostaglandin, the author has endeavoured in this investigation to make quantitative determinations of the total content of contractive substance in sperm from different individuals.

The material was obtained from the sperm laboratory at the Gynaecological Clinic of the Karolinska Sjukhuset and consisted of 155 specimens of sperm. It is to be noted in this connection, therefore, that these specimens do not constitute a normal material but were taken from men whose marriages were childless. It would naturally have been desirable to obtain, if it had been possible, a considerable number of specimens of sperm from demonstrably fertile men, but in practice this did not prove possible.

The method employed for the quantitative determination of the content of contractive substance is the same in principle as that employed for the standardization of preparations of hypophysis, *i. e.* comparisons on the test object in vitro of the effect of a known quantity of prostaglandin and a certain quantity of sperm with an unknown content of contractive substance. By way of experiment a rat uterus was first used as the test object, but it did not prove to give constant results for the same dose of prostaglandin. The classic test object — guinea-pig uterus — also proved to be unusable, as it relaxed all too slowly.

In its place the author used rabbit intestine, and this proved to be very suitable for the purpose. The animals were killed by means of air emboli, after which a piece of duodenum and jejunum one decimetre long was immediately excised, washed with Tyrode's solution and then placed in a dish of this solution at room temperature, where it was left for $\frac{1}{4}$ — $\frac{1}{2}$ hour before it was used. Then a piece approximately 2 cm. in length was placed in an intestinal bath, which was filled with Tyrode's solution and carbogen gas (consisting of 95 % O_2 and 5 % CO_2) bubbled through, and the whole kept at a temperature of 37—38°. The volume of the intestinal bath was 25 ml. The movements of the intestine were then registered kymographically. Only such pieces of intestine as exhibited a regular motility with equal amplitudes after a short time were accepted. The standard employed was a prostaglandin solution, produced in pure form by Professor U. S. v. EULER, to whom I now proffer my thanks for placing it at my disposal and for the valuable advice which he has given me. Titration was not begun until it had been ascertained that the same quantities of prostaglandin gave practically the same quantitative indications. The sperm specimens were centrifuged for about 5 minutes, after which the cell-free plasma was usually diluted with 9 parts of Tyrode's solution. The quantity of prostaglandin which, as a rule, gave the most appropriate indications was 0.1 ml. of a standard solution containing 1 unit of prostaglandin per ml. One unit of prostaglandin has been defined by v. EULER as the quantity which lowers the blood pressure in rabbits by 25 %. Titration was then carried out in such a way that by means of the injection syringe there were added to the intestinal bath a certain fixed quantity of prostaglandin (*i. e.* in most cases 0.1 unit) alternating with varying amounts of the specimen until equal quantitative results were obtained. Every time the increase in tonus reached its maximum the kymograph was stopped and the fluid in the intestinal bath changed, after which a pause of 5 minutes was made.

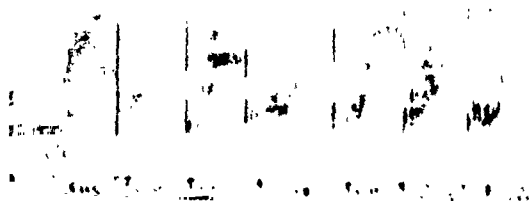


Fig. 1.

In most cases the content of contractive substance could be determined very satisfactorily. Fig. 1 shows a typical titration curve of this kind. In a large number of cases, however, it proved that the shape of the curve which was obtained when sperm was added diverged completely from the prostaglandin curve, as is seen in fig. 2. In such cases a very rapid, almost tetanic increase in tonus was obtained with very

small initial amplitudes, which subsequently increased with a simultaneous lowering of tonus, in contrast to the even increase in tonus obtained with prostaglandin. In such cases it is naturally very difficult to indicate an exact value for the content of contractive substance, and one has to content oneself with a probability value within fairly wide limits. In about 50 of the cases this effect was so pronounced that it was quite impossible, on the whole, to indicate any definite value. These specimens have, therefore, not been included in the material, but they are of interest in that they show that there are contractive substances in the sperm other than prostaglandin.



Fig. 2.

In some cases determinations were made both on atropinized and non-atropinized specimens of the same intestine, lower values throughout being obtained in the former case. This must be interpreted as a proof of the presence of choline in the sperm, the effect of which had thus disappeared owing to the atropinization. On the other hand, even in certain of these experiments on atropinized intestine, the characteristic tetanic tonus increase just referred to is met with, and therefore it is probably conditioned by some substance other than choline.

In order to obtain some conception of the certainty of the method, 31 double determinations were made, and the results have been worked up statistically. As was expected, it then proved that the mean error is as great as 20—25 %. Nevertheless, the method will probably afford a certain conception of the content of contractive substances in different specimens of sperm.

As a rule the determinations were made on the same day as the specimens were delivered to the sperm laboratory, in some cases, however, not until the following day, as it proved that storage in a refrigerator for 24 hours did not change the content to any appreciable extent. Unfortunately, only in exceptional cases was it possible to obtain specimens from the same person on several occasions, and therefore no pronouncement can be made as to the degree to which the content of contractive substance varies on different occasions.

The results of 155 determinations are shown in table I. It was possible to prove contractive substance in all the specimens, and

Table 1.

	<5	5—10	10—15	15—20	>20
Prostaglandin equivalents					
Number of samples	63	45	18	20	9
Per cent	40.6	29.0	11.6	12.9	5.7

in nearly half of them the content was found to be less than 5 prostaglandin equivalents. The mean value was 8.3 equivalents.

The results obtained have been correlated with the other properties of the specimens of sperm, such as the size of the specimen, the number of spermatozoa, the percentage figure of motile spermatozoa, the degree of motility, and the lifetime and morphological appearance of the spermatozoa,¹ but it was not possible to prove any connection between the content of contractive substance and these factors. Thus the 10 cases of aspermia in the material exhibit the same varying content of contractive substance as the sperm specimens with the normal number of spermatozoa, and in the 35 specimens which were examined in respect of motility within 2 hours after ejaculation and exhibited more than 50 % of motile spermatozoa, the same distribution of the amount of contractive substance is met with as in the whole material.

Discussion.

The investigations confirmed the observations of v. EULER, GOLDBLATT and others that the sperm plasma from man has a contractive effect on smooth musculature. However, this characteristic proved to be due to the occurrence, not only of prostaglandin and choline, but of at least one other substance, the nature of which has not been ascertained more closely, except that it is unaffected by atropine. The fact that sperm contains a number of substances that have a contractive effect on smooth musculature naturally renders the determination of the total content of contractive substance in the individual specimen more difficult, in the first place as it is not known how and to what extent these different substances change during the time immediately after ejaculation. It is known, *e. g.* from KAHANE and LEVY's investigations (1937) that the choline content in sperm increases continuously during the first 10—12 hours after ejaculation, and these authors would explain this on the assumption that the choline is present in a precursor form, which after ejaculation is

¹ These particulars were courteously placed at my disposal by the head of the sperm laboratory, Dr Eric Nordlander, to whom I herewith proffer my thanks.

transformed into choline by some enzymatic process. It is of course possible that the other contractive substances also undergo similar changes. In the second place it is not known to what extent the different substances may counteract or reinforce each other in respect of the contractive effect. In order to elucidate these problems it will probably be necessary to isolate the various substances and determine their relative amounts in the individual specimens. As regards what is in all probability the most important factor, prostaglandin, a method of extraction is being worked out. Even apart from these objections it will be possible to say, however, that the content of contractive substance varies somewhat considerably in different individuals. Therefore it is the author's intention to attempt, if possible, to investigate to what extent these variations may be conceived as having any influence on uterine and tubar motility in the case of conception.

Summary.

1. The total content of contractive substance in human sperm was determined from 155 specimens of sperm by titration on rabbit intestine *in vitro*, with prostaglandin as the standard. The content varied greatly in different specimens, but contractive substance was met with in all of them.

2. In certain specimens the factor which is active on rabbit intestine consists — apart from prostaglandin and choline — of at least one other substance, which leads to a very rapid increase in tonus and is indifferent to atropine.

3. There is no correlation between the total content of contractive substance and the motility and life of the spermatozoa.

This investigation was made possible thanks to a grant from the special fund for medicine of the the Nobel Foundation.

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From the Gynaecological Clinic of the Karolinska Sjukhuset.

Some Preliminary Experiments in Connection with the Effect of Prostaglandin on the Uterus and Tubae in Vivo.

By

JAN ASPLUND,
Stockholm.

Received 19 October 1946.

A fact which has been known for a considerable time is that human prostate secretion and sperm plasma from man have a contractive effect on smooth musculature (OTT and SCOTT, 1909; BATTEZ and BOULET, 1913; MACHT and MATSUMOTO, 1920), but it was the investigations of GOLDBLATT (1933, 1935) and v. EULER (1934, 1935, 1936, 1939) which first made it seem probable that this property was largely due to the presence of a lipoidsoluble depressor substance which, in respect of its biological and chemical properties, differed from all previously known depressor substances. This substance, however, which v. EULER called prostaglandin, could only be proved in the secretion from glandula vesicularis from sheep, while the sperm plasma from other animals investigated proved completely inactive.

As it is conceivable that contractive substances in the sperm may be of a certain importance for the transport of spermatozoa, the present author carried out a number of preliminary investigations of the effect of sperm plasma and pure prostaglandin on the uterus and tubae in vivo and the resorption conditions of the active factor, with different modes of application.

As far as the present author has been able to find, the effect of prostate secretion on the motility of the uterus in vivo has previously only been subjected to investigation once, namely by BARNES (1939), who introduced into the vagina of rats mixtures

of methylene blue and prostate secretion obtained by means of prostate massage. In post mortem examinations of the animals after varying intervals he was unable, however, to prove any propagation of the colour up into the uterus. Similar experiments with roentgen contrast medium also gave negative results. Further, he registered the uterine motility in the vagina and uterus by means of balloons introduced with a cystoscope. In this connection intravenous supplies of prostate secretion had very little effect on the uterine motility and no effect on the contractions in the vagina. From these experiments BARNES drew the conclusion that prostaglandin is of no importance for the transport of the spermatozoa from the vagina to the uterus and through the uterus.

The present author's attempts to study the effect of sperm and prostaglandin were carried out exclusively on rabbits. The prostaglandin employed was produced from human sperm at the Physiological Institution at the Karolinska Institutet in accordance with the extraction method worked out by v. EULER. In the experiments made with native sperm plasma, specimens of sperm — which were at most 12-hours old — from the sperm laboratory of the Gynaecological Clinic at the Karolinska Sjukhuset were used, and before the experiment these had been freed from spermatozoa by means of centrifugation. For registering the uterine motility the following experimental arrangements were employed: Under urethane narcosis the abdomen was opened and the small intestine was pushed up behind and above a loop of colon, which, by fixation to the abdominal wall in a couple of places, prevented the small intestine from passing down into the lower part of the abdominal cavity, which thus then contained only the vagina and uterus with its adnexa and the rectum. The distal part of one uterus horn was then fixed to the lateral wall of the abdomen. In the incision in the median line was then sewn an oval piece of celluloid with a hole approximately 2 cm. in diameter, after which the abdominal cavity was filled with Tyrode's solution at body temperature. The movements of the uterus were then registered on a kymograph with a well-balanced pen which was fastened to the uterus horn by means of a thread over a pulley wheel inserted in the celluloid frame. In the majority of cases a fairly good registration of the longitudinal contractions in the uterus was obtained, by this method. In certain cases registration of the blood pressure was made at the same time.

With intravenous or intraperitoneal supplies of prostaglandin a very clear and rapidly onsetting effect on the uterine motility was obtained, as appears from fig. 1. The effect manifested itself in the first place in a rapidly onsetting increase in tonus, at first with small amplitudes, which became larger and larger, however.

The magnitude and duration of the effect depended upon the amounts of prostaglandin supplied. When the blood pressure was registered, it could be established that the effect on the uterine motility came at approximately the same time as the fall in blood pressure, but the lowered blood pressure persisted longer than the increased uterine motility, which in no case lasted longer than 15—20 minutes. In one case when a second injection of prostaglandin was given, before the blood pressure had had time to return to normal, the effect on the uterus was considerably

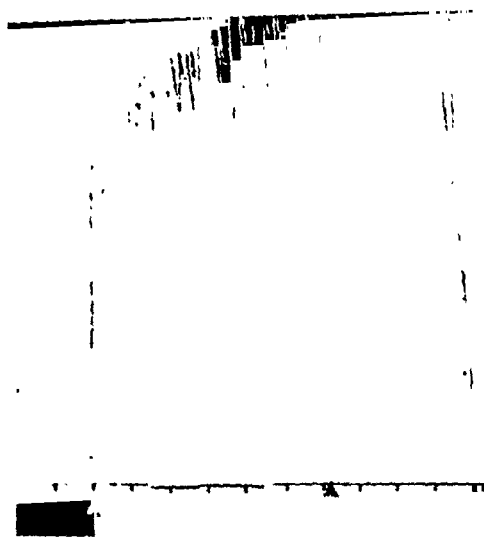


Fig. 1. The effect of prostaglandin on the rabbit's uterus in vivo with intraperitoneal application. Time-registration every minute.

less pronounced than with the first supply. Intravenous and intraperitoneal supplies of sperm had the same manifest effect on the uterus.

With the object of investigating the resorption conditions of the prostaglandin with different methods of application, a series of experiments was carried out with registrations of the blood pressure. In this connection it was presupposed that the factor which lowered blood pressure and that which had a contractive effect on the uterus was one and the same substance, as had been maintained by v. Euler but disputed by Goldblatt. Registration of the blood pressure gives a much safer measure of the prostaglandin effect than the relatively rough mechanical registration of the uterine motility. The blood pressure was registered in the

ordinary manner by means of a cannula in a. carotis with a mercury manometer. In all the cases the animals were under urethane narcosis. As had been expected the most rapid and most pronounced fall in blood pressure was obtained with intravenous application, but with an intraperitoneal supply also a very rapidly onsetting effect appeared. When prostaglandin was injected directly into one uterus horn, there also appeared — though after a couple of minutes' latency — a clear fall in blood pressure. In a couple of cases a small catheter was inserted into the vagina, which had previously been ligatured immediately below cervix uteri, so that the prostaglandin injected through the catheter could not pass up into the uterus. In these experiments also a clear, though slowly onsetting, lowering of the blood pressure was obtained. As a control, all these experiments were initiated with a supply of Tyrode's solution, but no change in blood pressure could then be established. Thus these experiments prove that prostaglandin, or at least the blood pressure reducing factor in it, is resorbed via the uterus and vagina.

Further, some experiments were carried out with the object of studying the effect of the prostaglandin on the tubae in vivo. The insufflation method worked out by RUBIN (1927) was employed, with the assistance of a modification of Rubin's apparatus constructed by WESTMAN and PETERSEN (1945). In principle the method is to allow a steady current of CO_2 to pass through the tuba and to register the resistance to the current due to contractions of the tuba. The experiments were carried out as follows: In a rabbit under urethane narcosis a small hole was burnt with a thermocautery in the wall of the uterus, right up against the transition to the tuba. In this hole was inserted a small glass cannula, the point of which was inserted into the tuba and fixed so that it was air-tight. The cannula was then connected by means of vacuum tubes to the carbon dioxide container in the insufflation apparatus. When the current of carbon dioxide was turned on, it passed through the tuba and out through its abdominal ostium, CO_2 bubbles being observable in the bursa ovarica. The initial resistance in the abdominal ostium, which had to be overcome, varied from animal to animal, but for the most part it was in the neighbourhood of 50 mm. Hg. In some experiments, with the object of being able to register the blood pressure at the same time, and also of obtaining a still more sensitive registration of the tonus conditions of the tubal ostium, the capsule built into

the apparatus was replaced by a small Marey's capsule, which traced on a smoked drum.

With supplies of prostaglandin the effect shown in fig. 2 was obtained. Almost simulatenously with the fall in blood pressure the tonus fell noticeably in the tubal ostium; and at the same time there was a marked decrease in the amplitude. This effect persisted for approximately the same time as the fall in blood pressure, and presented itself clearly, whether the prostaglandin was administered intravenously, intra-periotoneally, intra-uterin-ally or intravaginally, although the effect was more marked with the first two methods. The same results were obtained with administration of native sperm.

Fig. 2. The effect of prostaglandin on the tonus of the abdominal tubal ostium and on the blood pressure with intravenous application. Time-registration every minute.

Discussion and summary.

These investigations, which can only be regarded as preliminary, proved that sperm and prostaglandin, the substance contained in it, exercise a manifestly stimulating effect on uterine musculature in vivo. Thus this is at variance with Barnes' results. In this connection, however, it must be taken into account that with his method of investigation he registered in the first place the circular musculature. It may also be questioned whether the introduction of balloons by means of a cystoscope does not lead to a considerable lessening of the motility.

Further, the present author's own investigations showed that to some extent the factor in prostaglandin which reduces blood pressure is resorbed already by the vagina and uterus, and therefore it is very probable that the uterus-stimulating factor may

also be resorbed there, although this could not be proved with the relatively rough registration of the motility employed.

Finally, the experiments show that sperm and prostaglandin cause a clear and fairly protracted lowering of tonus in the abdominal tubal ostium.

Both the established stimulation of the uterine musculature and the changes in the contraction conditions of the tubal ostium may conceivably be of importance for the transport of spermatozoa, a question which will be more closely studied in a later work.

This investigation was made possible thanks to a grant from the special fund for medicine of the Nobel Foundation.

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The Formation Mechanism of Oestrogenic Hormones.

I. The Presence of an Oestrogen-Precursor in the Rabbit Ovary.¹

By

LENNART CLAESSION and NILS-ÅKE HILLARP.

Received 23 October 1946.

Our knowledge of the biosynthesis of oestrogenic hormones is still not very extensive. There is a theoretical possibility that they are derived from cholesterol (FIESER, 1937) but hitherto no direct proofs of this theory have been advanced (SCHWENK, 1944, SALTER, 1945, PINCUS, 1946). A solution of the problem of this biological synthesis would be within reach if the precursor of the oestrogenic hormones could be demonstrated in the ovary. By means of polarization optical and histochemical methods, we have therefore investigated the possibility to demonstrate the presence of a substance, which is engaged in the intracellular metabolism of oestrogenic hormones in the form of oestrogen-precursor. This would make it possible to analyse directly both the production and the secretion of oestrogenic substances in the ovary.

Material and methods.

The material here investigated consists of ovaries from female rabbits, taken firstly during various sexual phases immature, anoestrous, oestrous, pseudo-pregnant, and pregnant animals, secondly from oestrous females at various points of time after coitus, and thirdly

¹ Aided by grant from Svenska Sällskapet för Medicinsk Forskning.

from pregnant females after administration of gonadotrophic hormone (pregnant mare serum gonadotrophins).¹

Frozen sections (10 μ) from formalin-fixed ovaries have been subjected to polarization optical and histochemical analysis (SCHULTZ' reaction (SCHULTZ, 1924), phenylhydrazine- and digitonin reaction). The following sterol reactions have further been performed on chloroform extracts of ovaries: LIEBERMANN-BURCHARDT, digitonin-, phenylhydrazine-reaction, ZIMMERMANN's m-dinitrobenzene reaction (ZIMMERMANN, 1935), ROSENHEIM's trichloro-acetic acid reaction (ROSENHEIM, 1929). Finally quantitative cholesterol determinations (according to GÖRTZ, 1934) have been performed on the ovaries; the main purpose of these determinations was to examine the parallelism between the amount of the polarization optically demonstrable substance and the cholesterol content.

The assessment of the quantitative variations of the demonstrated substance has been conducted with simultaneous polarization optical examinations and SCHULTZ' reactions. When applied to rabbit ovaries, both these methods yield the same result. To facilitate the assessment, sections from different parts of the ovary in all animals examined have been photographed under low magnification in polarized light. On the basis of these photographs it is then possible to compare directly the content of the demonstrated substance in the different ovaries. In the tables the approximative content, determined in this way, is denoted by + to + + + + + +; + + + + + + denotes the maximal storing in an ovary.

The oestrogenic activity of the demonstrated substance has been assayed — both before and after hydrolysis — on ovariectomized mice.

Qualitative Data.

Polarization-optical Characteristics.

Polarization optical analysis of rabbit ovaries (10 μ frozen sections) reveals that there exist intracellularly in theca interna and in the interstitial gland fine birefringent granulae which fill out the cells completely during certain functional phases of the ovary. Lipoid-solvents cause this birefringence to disappear completely. The double refraction is also abolished when sections are heated to 60° C. to return on cooling, when crystallization in the form of positive spherites takes place. These properties, characteristic for the granulae, suggest that they consist of cholesterol or of some related sterol.

¹ A. B. Leo, Hälsingborg, has kindly assisted us in our investigations by placing its preparation Antex Leo at our disposal.

Histochemical Reactions.

In order to prove the sterol nature of the birefringent substance the ovaries have been examined histochemically by means of SCHULTZ' reaction, which is positive in the presence of cholesterol (SCHULTZ, 1924, SCHULTZ and LÖHR, 1925, LAUX, 1926, KAUFMANN and LEHMANN, 1926). However, as this reaction is nothing but a modification of LIEBERMANN-BURCHARDT's or LIPSCHÜTZ' reactions, it is, as well as the two last-mentioned ones, nonspecific in so far as other related sterols, too, give positive responses. When applying SCHULTZ' reaction to ovarian sections which had been exposed to oxidation by means of ultraviolet light or ammonioferric sulfate, the birefringent granulae showed a strongly positive reaction: intensely green after treatment with ammonioferric sulfate, intensely reddish-violet after ultraviolet irradiation. These results are an additional support of the assumption that the granulae consist of cholesterol or of some related sterol.

When investigating the precipitability of the sterol with digitonin (0.5 per cent solution in 85 per cent ethyl alcohol) on ovarian sections, we found that the birefringent substance does not consist of free cholesterol. No formation of any crystallizing cholesterol-digitonid could be observed. Cholesterol esters on the other hand can naturally not be excluded through this reaction.

When applying BENNET's phenylhydrazine reaction (BENNET, 1940) to 50 μ thick frozen sections, a macroscopically observable faint yellow staining could be seen in the regions to which the birefringent substance was localised. As the formation of phenylhydrazones implies the presence of compounds with carbonyl groups, it seemed possible that the demonstrated sterol might consist of a ketosteroid. There is on the other hand no parallelism between the presence of the phenylhydrazone-forming and the birefringent substance. The result of the phenylhydrazone reaction is quantitatively the same whether the ovaries present a maximal storing of, or are completely emptied of sterol granulae. This result shows that the demonstrated sterol is not identical with the hydrazone-forming substance, and thus probably no ketosteroid.

Experiments with Sterol Reactions on the Extracted Substance.

Various reactions have been applied to chloroform extracts of ovaries with maximal storing of sterol granulae. The ovaries have

been cut into frozen sections, about 30 μ thick, and the sections extracted for 5 minutes with 10 ml chloroform per ovary. This treatment causes the lipid birefringence in the ovarian sections to disappear completely.

As the polarization optical and histochemical investigations indicate that the birefringent substance consists of cholesterol or of some related sterol, quantitative cholesterol determinations were performed on ovaries either with maximal storing of, or emptied of sterol granulae. In this way it may be possible to decide whether there is a parallelism between the presence of birefringent substance and of cholesterol. — For these experiments we used pregnant female rabbits (See table I) the ovaries of which showed a maximal storing of sterol. One ovary was extirpated after which pregnant mare serum gonadotrophins were injected intravenously. The treatment with gonadotrophins causes a mobilization of the birefringent substance in the remaining ovary (See page 126). This ovary was removed 19 to 24 hours after the injection. The total cholesterol content was determined colorimetrically on chloroform extracts from the ovaries (after removing corpora lutea) by means of the acetyl chloride reaction according to GÖRTZ (1934). From each ovary some 10 μ thick frozen sections were prepared for polarization optical examination of the birefringent substance.

As seen from Table I ovaries from pregnant untreated animals have a high total cholesterol content. The polarization optical investigation of these ovaries shows that there is a very extensive storing of birefringent substance. After treatment with gonadotrophic hormones, on the other hand, the ovaries show a low cholesterol content and none, or very little, birefringent substance. There is thus a parallelism between the total cholesterol content and the presence of birefringent sterol in the ovaries. This parallelism indicates that the latter substance is identical with cholesterol. The quantitatively large amounts of cholesterol in the untreated animals also suggest that such must be the case, as so large cholesterol amounts as these must make themselves visible, polarization optically as a great massing of birefringent substance.

In order to determine whether the demonstrated sterol is present in free form, or bound in the form of ester, the amount of ester cholesterol has been determined in six ovaries after digitonin precipitation of the free cholesterol. As shown in Table I the main part of the cholesterol is present in the form of ester. This result

Table I.

Comparison between the amount of polarisation optically demonstrable sterol and the amount of cholesterol in rabbit ovaries before and after gonadotrophic stimulation. One ovary was extirpated at the same time as pregnant mare serum gonadotrophins were administered, the other was extirpated 19 to 24 hours after the injection. The amount of cholesterol is given in mg per ovary as well as per g ovarian tissue.

Animal No.	Day of Pregnancy	Number of Hours between Extirpation of the two Ovaries	Amount of Injected Pregnant Mare Serum Gonadotrophins in I. U.	Weight of the Ovaries in g (without Corpora lutea)	Amount of Polarisation Optically Demonstrable Sterol	Total Amount of Cholesterol		Ester Cholesterol	
						in mg per Ovary	in mg per g Ovarian Tissue	in mg per Ovary	in mg per g Ovarian Tissue
OK 250	5	24	225	R 0.427 L 0.546	Not examined	6.9	16.0	0.5	11.1
OK 247	6	24	300	L 0.570 R 0.630	+ + + + + + + + + +	3.4 9.1	6.4 16.4		
OK 248	6	24	300	R 1.196 L 1.705	+ + + + + + + + + +	3.7 13.4	5.9 11.6		
OK 255	7	24	225	R 0.275 L 0.415	+ + + + + + + + + +	3.8 5.5	2.2 20.0	3.6	13.1
OK 256	7	24	225	L 0.315 R 0.515	+ + + + + + + + + +	2.3 11.9	5.5 37.5	1.9 8.9	4.6 28.3
OK 249	13	19	300	R 0.539 L 1.313	0 + + + + +	2.1 21.9	4.1 29.6	1.8	3.5
OK 251	13	19	300	R 0.292 L 0.463	0 + + + + +	1.7 8.1	1.4 27.7	6.1	20.9
						1.9	4.7		

explains the negative digitonin reaction obtained on ovarian sections.

After desiccation of chloroform extracts from pregnant ovaries (without corpora lutea) and saponification with alcoholic NaOH solution a large precipitate was obtained with digitonin. When this precipitate had been filtered off, the extract did no longer yield a positive response to LIEBERMANN-BURCHARDT's reaction. This shows that the demonstrated chromogenic sterol is precipitable with digitonin.

Ovarian extracts have also been tested with the phenyl-hydrazine reaction (0.2 per cent 2, 4-dinitro-phenylhydrazine in 2 N HCl), with ZIMMERMANN's m-dinitrobenzene reaction (ZIMMERMANN, 1935) and with ROSENHEIM's trichloro-acetic acid reaction (ROSENHEIM, 1929). In all cases the results were negative. The reactions were performed on the saponified extract, and the following sterol amounts were employed (counted as cholesterol in the quantitative cholesterol determinations): 450, 3,000 and 350 γ respectively.

Experiments with the Oestrogenous Activity of the Extracted Sterol.

Although the investigations reported above indicate that the demonstrated sterol is identical with cholesterol or related to this substance, we considered it necessary to complete the results obtained with an examination of the oestrogenic activity of the sterol. For this purpose we used extracts containing the amount of sterol in $\frac{1}{3}$ to 1 ovary (without corpora lutea) from pregnant rabbit females. The extracts were tested on ovariectomized mice. Both before and after hydrolysis the extracts were, however, found to be quite inactive.

Quantitative Data.

We have analysed the quantitative variations of the demonstrated sterol during the various sexual phases in order to determine the connection of this sterol with the formation and secretion of the oestrogenic hormones. Our investigations of rabbit ovaries have led to briefly the following results:

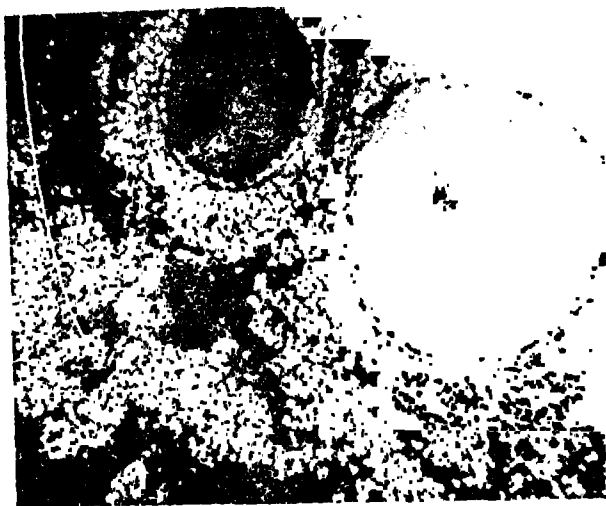


Fig. 1. Ovary from oestrous rabbit female (OK 167). A great amount of birefringent sterol is observed in the cells of the well-developed interstitial gland. 10 μ frozen section through the cortex region with two large follicles. Crossed Nicols. $\times 30$.

1 a) Ovaries from Oestrous Females.

The fact that the animals in this group (See table II) were in oestrous, was determined partly by their readiness to mate, partly on the basis of the appearance at section of their uterus and ovaries. The animals were killed immediately after coitus.

In the well-developed interstitial gland of these ovaries a large amount of sterol is generally found (Fig. 1). The cells are more or less filled with sterol granulae; these granulae are however often less frequent in the interstitial cells of the medulla. An approximate assessment of the amounts of sterol observed is found in Table II.

1 b) Ovaries from Oestrous Females post Coitum.

The animals in this group (Table III) were killed $\frac{1}{2}$ to 26 hours post coitum.

The animals that were killed $\frac{1}{2}$ to 2 hours post coitum still show a relatively large amount of sterol in the interstitial gland. As early as 3 hours post coitum (Fig. 2), however, a marked change can be observed. Sterol granulae are mobilised from the cells, and 5 hours p. c. and later it is only with difficulty or not

Table II.

Amount of polarisation optically demonstrable sterol in oestroues, anoestrous and immature females.

Animal No.	Amount of polarisation optically demonstrable sterol
Oestrous:	
OK 129	++++
OK 153	++++
OK 154	++
OK 167	+++
OK 206	++++
OK 227	++++
OK 228	++++
OK 229	++++
Anoestrous:	
OK 152	++
OK 174	+
OK 181	++
OK 195	++
Immature:	
OK 122	0
OK 185	+
OK 186	+
OK 187	+

Table III.

Amount of polarisation optically demonstrable sterol in oestrous females $\frac{1}{2}$ to 26 hours post coitum.

Animal No.	Number of hours post coitum	Amount of polarisation optically demonstrable sterol
OK 155	$\frac{1}{2}$	++++
OK 204	1	++++
OK 203	2	+++
OK 205	3	0
OK 235	3	+
OK 207	4	++
OK 196	5	+ (left ovary)
OK 236	$5\frac{1}{2}$	+
OK 175	6	+
OK 238	$7\frac{1}{2}$	0
OK 240	8	0
OK 176	9	+
OK 196	9	+ (right ovary)
OK 243	$9\frac{1}{2}$	0
OK 239	$10\frac{1}{2}$	0
OK 242	26	+



Fig. 2. Ovary from oestrous rabbit female 3 hours post coitum (OK 205). The cells of the interstitial gland are quite emptied of birefringent sterol granulae. Small amounts of birefringent substance can be observed intercellularly. 10 μ frozen section through the cortex region with one large follicle. Crossed Nicols. $\times 30$.

at all possible to demonstrate any sterol either polarisation optically or by means of SCHULTZ' reaction (See Table III).

2) Ovaries from Anoestrous Females.

The fact that the animals in this group (Table II) were in anoestrous, was determined by the appearance at section of their uterus and ovaries.

In this group only a small number of sterol granulae can be observed in the cells of the well-developed interstitial gland.

3) Immature Females.

The sterol amounts here observed are still smaller than in the anoestrous adult animals. The interstitial gland is rather inconspicuous and contains either no sterol granulae at all or only very few of them (See Table II).

4 a) Ovaries from Pregnant and Pseudo-Pregnant Females.

The whole ovary, from the hilus region to the capsule, is filled with interstitial cells, in all of which great amounts of sterol

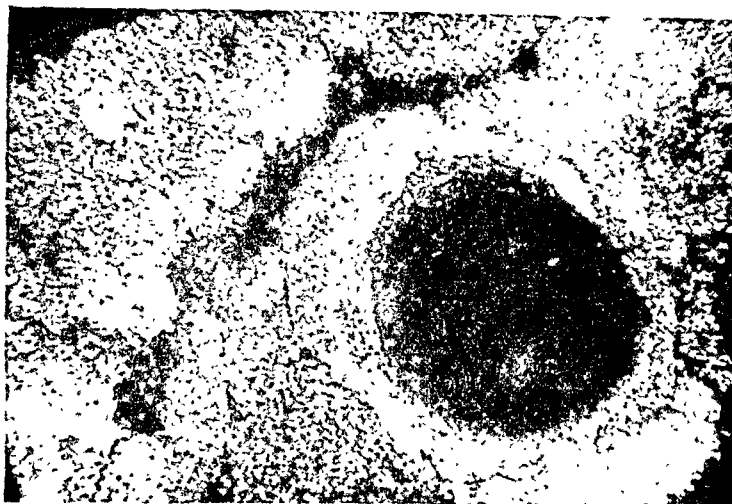


Fig. 3. Left ovary from pregnant (19th day) rabbit female (OK 192). The ovary was extirpated immediately before an injection with gonadotrophic hormone. Great amounts of birefringent sterol in the interstitial gland. 10 μ frozen section through the cortex region with one large follicle. Crossed Nicols. \times 30.

granulae are found (Fig. 3). The storing of the sterol, mobilised after coitus, takes place fairly rapidly, so that already 2 to 5 days p. c. approximately the same amounts of sterol are found as those observed in the oestrous animals (Table IV). Later on during pregnancy the storing increases further still. From the seventh day of pregnancy onwards the amounts of sterol are conspicuously greater than in the oestrous females; after this time the amounts remain the same during the rest of pregnancy.

4 b) Ovaries from Pregnant Females Treated with Gonadotrophic Hormone.

To be able to compare directly the amount of sterol in one and the same animal before and after treatment with pregnant mare serum gonadotrophins (50—300 I. U. Antex Leo intravenously) one ovary was extirpated immediately before the injection. The remaining ovary was extirpated at various points of time up to 72 hours after the injection (See Table V).

In the same way as coitus, the treatment with gonadotrophic hormone causes a mobilisation of the sterol in the ovaries. In all the animals — except OK 194 — the sterol was mobilised to such a great extent that it could not at all or only very sparingly be

Table IV.

Amount of polarisation optically demonstrable sterol in pregnant and pseudopregnant females.

Animal No.	Day of Pregnancy	Amount of polarisation optically demonstrable sterol
OK 200	2	+++
OK 230	2	++
OK 131	5	++
OK 231	5	++++
OK 173	6 (pseudopregnant)	+++++
OK 118	7	+++++
OK 170	12	+++++
OK 178	13	+++++
OK 180	14	+++++
OK 179	16	+++++
OK 220	30	+++++
OK 216	? (pseudopregnant)	+++++
OK 215	?	+++++

Table V.

Amount of polarisation optically demonstrable sterol in pregnant ovaries before and after gonadotrophic stimulation. One ovary was extirpated at the same time as pregnant mare serum gonadotrophins were administered, the other ovary was extirpated 19 to 72 hours after the injection.

Animal No.	Day of Pregnancy	Number of hours between extirpation of the two ovaries	Amount of injected pregnant mare serum gonadotrophins in I. U.	Amount of polarisation optically demonstrable sterol
OK 247	5	24	300	Left ++++ Right +
OK 248	6	24	300	Left ++++ Right +
OK 255	7	24	225	Left +++ Right +
OK 256	7	24	225	Left ++++ Right 0
OK 188	9	48	100	Left ++++ Right +
OK 194	12 (pseudo-pregnant)	24	50	Left ++++ Right ++
OK 249	13	19	300	Right ++++ Left 0
OK 251	13	19	300	Right ++++ Left 0
OK 177	16	48	200	Left ++++ Right 0
OK 189	16	72	200	Left ++++ Right +
OK 192	19	24	100	Left ++++ Right +
OK 190	29	24	100	Right ++++ Left +

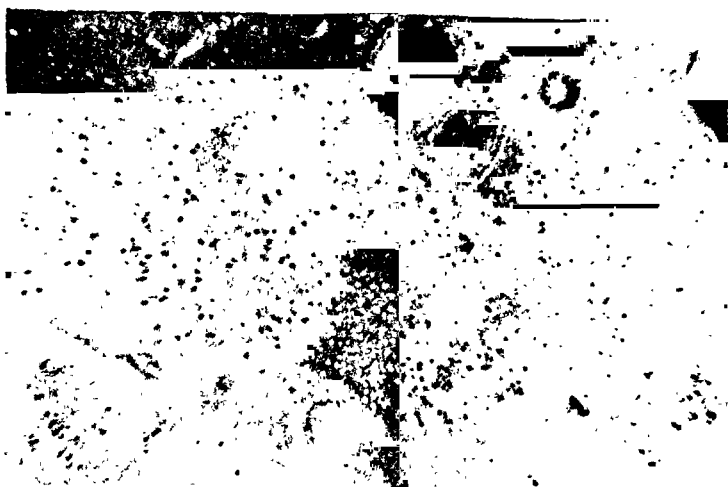


Fig. 4. Right ovary from the same animal as in Fig. 3. The ovary was extirpated 24 hours after an injection of 100 I. U. pregnant mare serum gonadotrophins. The interstitial gland is completely emptied of birefringent sterol. Some small amounts of birefringent substance can be observed intercellularly. 10 μ frozen section through cortex and medulla. Crossed Nicols. $\times 30$.

observed under the polarisation microscope (cf. figs. 3 and 4) or by means of SCHULTZ' reaction. The experimental animal OK 194 did not receive more than 50 I. U. Antex Leo, however, which may explain why the mobilisation in this case was not so extensive as in the other animals.

Quantitative cholesterol determinations were also performed on the ovaries of six animals reported in Table V (See table I). The differences in the amount of chromogenic sterol before and after the injection of pregnant mare serum gonadotrophins also illustrate the capacity of the gonadotrophic hormones to mobilise the observed sterol.

The sterol we have found thus shows quantitative variations during the different sexual phases. In ovaries under minimal gonadotrophic influence (immature, anoestrous) the substance can either not be found at all or only very sparingly. The oestrous and pregnant ovaries on the other hand present a great amount of stored sterol in the well-developed interstitial gland. At rapid and intense gonadotrophic stimulation of these latter ovaries the sterol is mobilised to such an extent that it can either not at all, or only to very little extent be demonstrated polarisation optically or histochemically.

Discussion.

Our investigations, reported above, have led to the result that there occurs in the interstitial gland of the rabbit ovary a birefringent substance, the quantity of which varies according to the sexual phase. The polarisation optical properties and chemical reactions of this substance prove that it is a sterol. The cholesterol reactions here tested — SCHULTZ' reaction, LIEBERMANN-BURCHARDT, the acetyl chloride reaction — give positive responses. However, as these reactions are not specific for cholesterol, but give positive responses also in the case of related sterols, the substance which we have observed cannot without further proofs, be identified with cholesterol. That it nevertheless is of cholesterol type is indicated by its being precipitable with digitonin, which is a characteristic of sterols with a 3-hydroxyl group (FERNHOLTZ, 1935, REICHSTEIN, 1936). The other reactions which we have performed do not contradict such an interpretation. The negative phenylhydrazine reaction and the m-dinitrobenzene reaction, which latter is positive for steroids with $-CO-CH_2$ configuration (ZIMMERMANN, 1935, 1938, 1944, CALLOW, CALLOW and EMMENS 1938) indicate that the birefringent substance does not consist of a ketosteroid. Neither does the sterol give a positive ROSENHEIM reaction (with trichloro-acetic acid) which is characteristic for sterols with a system of conjugated double bonds, or with a double bond in 2, β -position to a hydroxyl group (SCHOENHEIMER and EVANS, 1936).

Already the presence of great amounts of the sterol here discussed in the ovary suggests that it is engaged in the intracellular metabolism of oestrogenic hormones. It is not very probable that so great amounts of sterol in the cells of the interstitial gland should be engaged in the general metabolism of these cells. Consequently it appears more probable that the sterol must be connected with the special function of these cells, viz. the production of oestrogenic hormones. Our investigations of ovaries from oestrous females post coitum and from pregnant females after treatment with gonadotrophic hormones support this view. After coitus and after injection of gonadotrophic hormone a forced secretion of oestrogens sets in. *The sterol is mobilised parallel with this production of active hormone.* When applying an intense stimulus to the ovary this mobilisation may be completed within the course of some hours,

and the amount of sterol may fall from high to very low values. *This suggests that the sterol which we have observed is intimately connected with the formation of oestrogenic hormones.*

The obtained results: the presence of a sterol of cholesterol-type, stored in the cells of the interstitial gland, which in itself is biologically inactive, and which is directly engaged in the formation of oestrogenic hormones, indicate that *this sterol is the precursor of the active hormone.* As this precursor can be demonstrated polarisation optically and histochemically, there is thus a possibility of analysing directly both the production and the secretion of oestrogenic substances in the ovary.

Summary.

By means of polarisation optical and histochemical methods we have succeeded in demonstrating the presence of a sterol of cholesterol type — probably identical with cholesterol — in the interstitial gland and in theca interna of the rabbit ovary. The amount of this sterol varies according to the sexual phase: during oestrous, pregnancy and pseudo-pregnancy it is found to be stored in the cells in large amounts, while the immature or anoestrous ovaries on the other hand show either none or only very small amounts.

The majority of the sterol is present in the ovary in the form of ester. It is biologically inactive.

In case of forced secretion of oestrogenic hormones (coitus, injection of gonadotrophic hormone) the sterol is mobilised from the ovaries. If an intense gonadotrophic stimulation is applied, this mobilisation may be completed within the course of some few hours, and the amount of sterol falls from high to very low values. This indicates that the demonstrated sterol is intimately connected with the formation of oestrogenic hormones.

The results obtained in the course of our investigations suggest that the sterol is the precursor of the oestrogenic substances formed in the rabbit ovary.

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On the Refractive Index of the Retina.

By

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When light passes from one medium to another there takes place at each separating surface, partly a reflection which causes a loss of light and partly a refraction which causes the rays to deviate from their course. The condition for this occurrence is that the media in question have different refractive indices.

The refractive media of the eye are generally considered to be cornea, aqueous humor, lens and vitreous. One more seldom remembers that also the retina, at least its 8 anterior layers, also should be included. The rods and cones, the light percipient elements of the retina, lie in the deepest layer of it, that is to say, next to the choroid. While the refractive indices of the first named media since long are known, the refractive index of the retina is more seldom mentioned in literature.

Every ophthalmologist can convince himself of the fact that the refractive indices of the vitreous and the retina are not equally large when, using an ophthalmoscope, he observes the lively regular reflection which takes place on the surface separating the said layers. Therefore, at least, the index for the Limitans Interna is not the same as for the vitreous. The activity of the reflection increases in the direction of the short wave end of the spectrum. From this it is possible for us to conclude that the dispersion in both of the said media is normal.

In his classic handbook on physiological optics, HELMHOLTZ says that the refractive index of the retina ought to be consider-

ably greater than that of the vitreous. GULLSTRAND also draws the same conclusion when he says that not only the index for Limitans Interna but also that of the other retinal layers should be considerably greater than that of the vitreous. The refractive index of the retina is also mentioned by other investigators but they deplore the fact that they don't know its value. The only one who seems to have undertaken the task of determining this, is NORDENSON. I will revert to his investigations later on.

The task is not so easy. The majority of methods of determining the refractive index in solid substances demand that the surface of the object is absolutely even or that its thickness is exactly known. Methods of this nature can therefore not be employed when it is the question about a tissue of so flaccid a consistence as the retina. In order to determine the index of the various tissues, SPALTEHOLZ has worked out a special method. This is based on the fact that the examined tissue reflects least light and is thus best transparent when it is saturated and surrounded by a solution, the refractive index of which is the same as that of the tissue. The disadvantage of this method is that it necessitates a fixed and dehydrated preparation. The value obtained under such conditions cannot be compared with the index which prevails in the living tissue.

When, last autumn, I began an investigation of the refractive index of the retina, I endeavoured to determine the index as near to the physiological conditions as possible. For this purpose I found it better to use *Pulfrich's* or *Abbe's* method. In the beginning it was my intention to investigate the refractive index for the Limitans Interna which, from a catoptrical point of view, is the most interesting retinal layer. But as the thickness of this tissue is only $2\ \mu$ the task proved too difficult. Under these circumstances I made the retina in its entirety, with the exception of the pigment epithelium, the object of my investigations.

As other methods of investigation proved to be unusable for my purpose, I carried out my experiments with Abbe's double refractometer, an apparatus which is intended for solutions only. This, however, proved to be suitable even for my purpose. The principle of this apparatus is that one allows the light to fall from an optically denser medium into the examined substance and then determines the angle of incidence in the limiting surface at the appearance of the total reflection. As the index sought for is a function of this angle, it can thereby be determined. (With re-

gard to the construction of this apparatus see special literature.) It is sufficient to say here that if one has several transparent substances simultaneously in Abbe's refractometer, each with its own refractive index, the apparatus will then *show the index for the lowest refractive medium.*

The Object and Its Preparation.

All the experiments carried out by me on the eyes of animals were made in the Laboratory of Stockholm's Slaughter House in Enskede. It was therefore possible to obtain the eyes needed for the experiments immediately after the animals had been slaughtered. The experiments were begun with the eyes of cows. The preparation was made in the following manner: the eyes were opened by a tangential incision through all the tissues in the neighbourhood of the Ora Serrata. By manipulating, the posterior side of the bulbus, the stump of the optic nerve or the muscle remnants were seized, and the vitreous was removed by overturning the eye. As a rule it came away easily, the younger the animal was, the more easily it loosened. If the vitreous did not fall out by its own weight or if its removal caused a retinal detachment, the specimen was rejected. When the vitreous had thus been removed, the eye was turned inside out and fastened with one or two needles around the pyramid of an ordinary pulp egg storer.



Fig. 1. Egg storer used in experiments referred to in the text.

The circumference of the pyramid corresponds exactly to the eye "cap" and the uneven surface of the pulp retains the eye admirably whilst the sample is being removed. (By means of many control tests it was possible to make sure that this treatment of the eye had not, at all events not to any considerable degree, any influence on the value of the refractive index of the retina.) After this an incision was made with a very thin spatula of aluminium through the retina. By means of an unserrated iris forceps the upper edge of the wound was easily grasped and lifted far enough away from the lower layer so that the aluminium spatula could be inserted between the retina and the choroid. The spatula was directed with suitable small movements farther and farther in between the membranes until, by this means, one obtained a sufficiently large piece of the retina which was subsequently detached

by a pair of small conjunctival scissors from the surrounding tissue and placed on the lower hypotenuse of the refractometer. Care was always taken to obtain preparations from the same part of the eye (within Tapetum Lucidum). In order that the retinal specimen could be easily removed from the spatula, the instrument was previously smeared with solid paraffin. (As the refractive index of the paraffin is greater than that of the retina it does not affect the experiment.) The transfer of the specimen from the spatula to the prism is easily done with the help of an iris spatula. After this the prism of the refractometer were connected, the preparation illuminated with daylight and the refractive index was read. The refractometer was controlled before each test series and the comparator adjusted usually with the help of a pearl of vitreous so that the chromatic aberration was nullified. The index thus obtained applied to yellow light, n_D , according to FRAUNHOFER.

If the preparation was successful, the limit between the dark and illuminated field in the instrument was sharp and straight. The line of limitation naturally cannot be compared in sharpness with that obtained by a homogeneous solution but when one considers how anisotropic such a retinal sample really is, the line is unexpectedly sharp.

Samples in which the retina was wrinkled or torn during the preparation were discarded. Similarly with preparations which showed air bubbles when placed between the prisms. The tests which were carried out showed that the retina dried so rapidly that the refractive index rose many units in the third decimal when the retina had been without the protecting vitreous for only a few minutes. At the same time the border line in the ocular became unclear. Therefore the condition for an accepted test was that the interval between the opening of the eye and the placing of the preparation between the prisms should not exceed 75 seconds. As drying could not be wholly avoided, despite the many different preventive steps, only one sample was taken from each eye.

Although the vitreous seemed to have come well away from the retina during preparation it often happened that traces of it still remained in the sample. If this vitreal remnant was sufficiently thick, the apparatus, according to its construction, gave the preparation the value of the vitreal index, $n_D = 1.336$. On the other hand if the residual vitreal layer was very thin, one could observe two border lines in the ocular: up to the weaker shadowed border line of the vitreous and down to the darker one of the retina (Fig. 2).

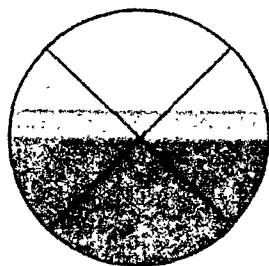


Fig. 2. Traces of vitreous create a second border line in the ocular.

Such tests were not accepted. (A similar vitreous border line usually disappears within one minute, at the most 2—3 min.) Likewise, preparations were always discarded if the line of demarcation for some or other reason, such as insufficiency in size of the sample, faulty placing of it on the hypotenuse and so on, was not clear enough to permit an easy and certain reading.

All the specimens were prepared within the first hour after the experimental animal had been slaughtered and they were always examined in a temperature of $+20$ degrees centigrade (Celsius). When, in winter time, the eyes were transported from the slaughter house to the laboratory they had to be protected against the cold because if they became chilled to a high degree or even froze, the refractive index often changed considerably in value. Prevailing atmospheric conditions and degrees of dampness were not taken into account. In order to remove any eventual "moment of inertia" the reading of the index value was carried out alternately from above and below. Of approximately 2,000 opened cow-eyes, 321 were passed as being satisfactory for experiment.

Despite the sorting out of the preparations and despite their rapid treatment, it is naturally impossible to compare the results obtained with conditions prevailing *in vivo*. In the retina, where all reactions happen quickly, the post mortem changes take place within a relatively short time and clouding of the tissues begins soon. Added to this that damages during preparation cannot be wholly avoided and that the sample is squashed between the prisms. All these circumstances alter the physiological refractive conditions prevailing in the tissues.

1. Falling Index Value.

In the beginning my technique was both slow and inexperienced: the line of demarcation between the illuminated and darkened ocular fields was therefore not sufficiently sharp. For this reason I made 10 readings for every retinal preparation. This showed that the index value at the end of each serial reading was regularly lower than at the beginning. In other words: *The refractive index of the retina fell whilst the preparation lay pressed between the prisms of the refractometer.*

This, then, is a singularly extraordinary phenomenon, quite contrary to what one might expect. When a piece of damp tissue is exposed to the action of the air, as is the case here, one has reason to expect a rise in the refractive index as the sample gradually exudes water. This also actually happens when examining other tissues, as for instance when determining the refractive index of the lens.

As my preparation technique gradually developed, the line of demarcation became so sharp that I was able to satisfy myself with one single focussing when reading. Afterwards it was even easy to follow more closely the said falling of the refractive index by regular readings, for instance, after every minute. This has also been carried out in the experiments described below.

A. Light Adapted Eyes Examined during the First Hour post mortem.

My many experiments have shown that no correlation exists between the freshness of the preparation and the degree of the falling in index value, provided it concerns eyes which have been examined during the first hour after slaughter. No greater post mortem changes could be noticed in the retina during this time. Neither had the age of the experimental animal any seeming influence on the value achieved.

The *falling* of the refractive index *always takes place*, and it begins already during the first minute, when it is usually greatest. In most cases the falling ceases 3 minutes, at the most 4 minutes, after the first reading. The lowest value reached remains constant for many hours.

Usually the index falls by 2—3 units of the third decimal and occasionally by even 4 units.

Table 1.

Variation of the index as function of time. Light adapted eyes.

	Minutes after the first reading									Remarks
	0	1	2	3	4	5	10	15	20	
Minutes post mortem	5	1.360	1.359	1.358.....						Age: 2 y.
	10	1.361	1.360	1.359	1.358.....					9 "
	15	1.362	1.360	1.360	1.359.....					9 "
	25	1.363	1.361	1.360.....						9 "
	40	1.360	1.359	1.358	1.357.....					8 "
	55	1.362	1.361	1.359	1.358.....					4 "
	90	1.359	1.358	1.357.....						3 "
	120	1.360	1.359	1.358.....						4 "
	150	1.358	1.357.....							9 "
	180	1.357	1.357	1.356.....						6 "
210	1.357.....					1.356.....			6 "	
240	1.358.....						1.357.....		2 "	
270	1.355.....								2 "	

(..... shows constancy of reading.)

Table 1 shows the variation of the index as function of time, and Fig. 3 shows a curve which was obtained from a material con-

sisting of 32 cases. In the latter figure the upper curve shows the rapidity of the fall whilst the lower one shows its amount. (Ordin. = units of the third decimal of the index value. Absc. = time in minutes.)

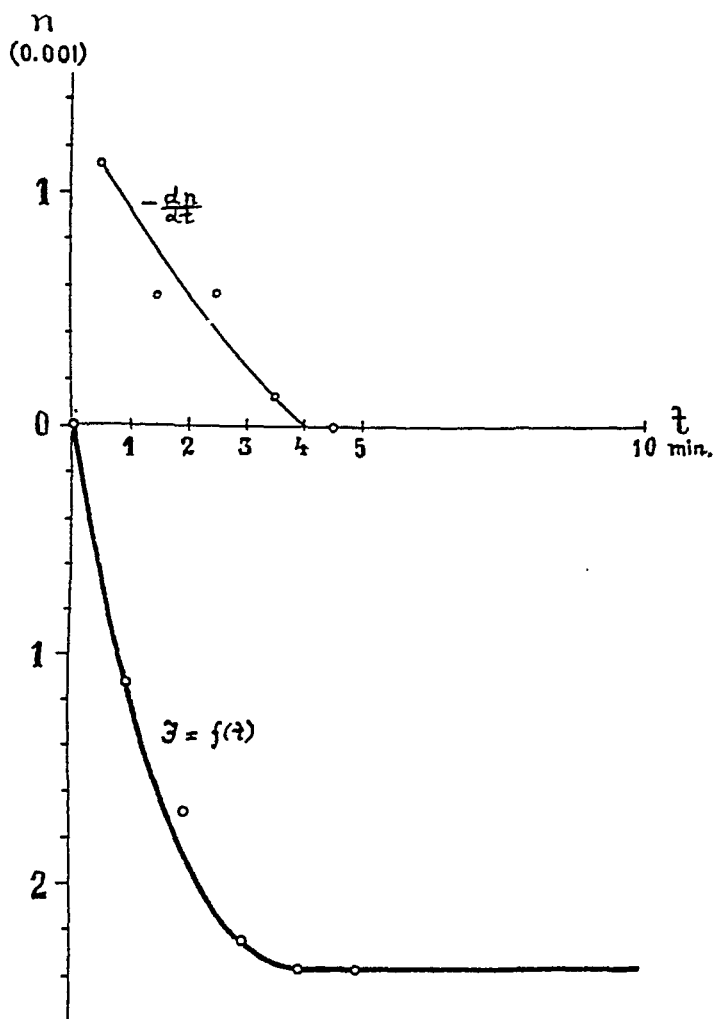


Fig. 3. The falling of index values in light adapted eyes (cows).

On what does the unexpected fall in the index value depend? Which layer in the retina gives rise to this phenomenon?

As was mentioned previously, we know that Abbe's refractometer from several samples lying simultaneously in the apparatus, gives the refractive index for the *lowest breaking*. On account of the different anatomical structures of the retina's separate layers,

these layers must probably also have different optical qualities and thus even different refractive indices. Thus it appears from the experiments carried out that the index for someone layer, eventually for several layers in the retina falls whilst the retina is pressed between the prisms of the refractometer. We suppose that the lowest breaking layer of the retina is in itself the cause of this extraordinary falling. When the falling is so rapid and so regular in its occurrence there seems here to be some reaction, yes, even one which is typical for the retina. From all accounts it is a question of a reaction whereby some part of the retinal layers *absorbs water*. Therefore it is a matter of finding out which of the retinal layers is the lowest breaking.

The only change in the retina known to us is that which takes place in the rod and cone layer in connection with the reaction of the visual purple.

All the examined eyes were adapted to light in the ordinary sense of the word. They were opened and examined in daylight. In order to attain definite knowledge as to how the visual purple or its variants had any part in this so-called index fall, the experiment was continued with cows' eyes which had been adapted to darkness.

B. Dark Adapted Eyes.

This part of the work proved, in practice, to be more difficult than was expected. Cows are not very easy to manage as experimental animals. At times my assistants were actually in danger of their lives. As there was no access to a room suitable for adaptation to darkness, the preparations and examinations were carried out in the following manner.

The cows were adapted to darkness by having their eyes bandaged. In the first place a large, convex, tightly-fitting black eye cap made of many layers of cloth was placed over the eye. This was fastened on all sides by covering it entirely with long, broad strips of adhesive tape. Over this a thick layer of cotton wool and the whole bound with a long gauze bandage wound round and round. Finally the head was covered with a thick "blind bandage" made of black leather which was drawn tightly with straps above and below the eyes so that it sat tightly round the head. The animals were then kept bound up in this manner for two hours after which they were slaughtered in the usual way. The head was removed and taken to a dark room where the band-

ages were removed and the eyes enucleated in red light. The retinal sample was obtained from the eye in the same manner as that in the previous experiment, in a temperature of $+20$ degrees Celsius, but naturally in red light ($> 615 \text{ m}\mu$). Whilst working in this contrast-poor light it proved to be especially difficult to obtain a successful preparation. When the preparation was placed in the apparatus and the prisms closed, light was passed through the refractometer from an electric lamp (60 ncp) with a ground glass and the index value read every half minute. Table 2 shows some curves thus obtained.

Table 2.

Variation of the index as function of time. Dark adapted eyes.

	Minutes after the first reading												Remarks
	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4	$4\frac{1}{2}$	5	10	
Minutes post mortem	5	1.357	1.360	1.359	1.358	1.357	Age: 5 y.
	5	1.358	1.359	1.360	1.359	1.359	1.358	2 "
	5	1.360	1.361	1.360	1.359	3 "
	15	1.358	1.360	1.359	1.359	1.358	4 "
	15	1.359	1.360	1.360	1.359	4 "
	35	1.359	1.360	1.360	1.359	1.358	4 "

(..... shows constancy of reading.)

Occasionally the refractive index remained unchanged during the whole of the observation time. Its value was usually 1.358.

We immediately observe a clear difference between these curves and those obtained in the experiments with light adapted retinae. The regular index fall in the light adapted retinae was absent in those adapted to darkness. The opposite occurs: the refractive index rises in the beginning of the observation series. In other words: at the beginning of the transillumination the lowest refractive layer of the retina now seems to *exude water* whilst the light adapted retinae, on the contrary, appear to absorb it.

Thus from the experiments referred to, it appears (1) that the refractive index of the light adapted retina was higher than that of the ones adapted to darkness, (2) that it always fell whilst the preparation lay in the refractometer and (3) that the refractive index of the to darkness adapted retinae on the contrary rose temporarily in the beginning or remained unchanged the whole time.

The results of my experiments thus support the previously stated supposition that the rod and cone layer is the lowest refractive layer of the retina and that visual purple really plays a part in the fluctuation of the index values.

How can one explain this occurrence?

As it is most probable that the changes are connected with visual purple and its variants we must give a short resumé of the composition of this substance as far as we hitherto know it.

KÜHNE and EWALD came to the conclusion that visual purple became bleached when subjected to heat. They compared this phenomenon with the coagulation of albumen in heat. Since then one has considered it probable that visual purple is a conjugated protein. This has been confirmed by many later investigators with, amongst other things, the help of absorption spectra. Visual purple which, according to BRODA, is probably a globulin, were combined with phospholipin in the retina.

One has carefully studied the process brought about by light in which visual purple passes over to visual yellow and visual white or, according to LYTHERGÖE, to indicator yellow. The latter term has, as a matter of fact, become more usual. For, as is now known, a solution of visual purple becomes either yellow or colourless on illumination, depending on whether the solution's reaction is acid or alkaline. Hence the term indicator.

According to MIRSKY (1936), bleaching ought to be the result of the denaturation of the protein molecules. WALD (1935) came to the result that visual purple molecules to begin with break up into a kind of carotene which he calls retinene, and this, subsequently, to vitamin A which should be the final product of breaking-up process. The process should be reversible so that the vitamin A could again be changed into visual purple. During this process vitamin A is lost and the amounts used replenished from the bloods depots. In this manner the hemeralopia occurring in the A-avitominosis gets its natural explanation. WALD's point of view has raised strong opposition, as it was not possible to prove the existence of vitamin A neither in the illuminated visual purple solution nor in the bleached retina. On the other hand, other carotenes have been encountered in the eye such as xanthophyll and taraxanthene.

The latest investigations in this matter which I have come across in literature gives, however, support to WALD's conception. Thus MORTON (1944) suggests the thought that the final

decomposition product of visual purple is composed of vitamin A aldehydes. For this reason one could not have been able to determine vitamin A in visual purple solution.

The structural composition of visual purple and its variants is not known. According to LYTHGOE it is possible that even indicator yellow could be a conjugated protein.

Without going into details about other works on this matter we might, by way of a summary of investigations, use LYTHGOE's words (1940): "We are justified in saying no more than that light weakens the chemical link which is responsible for the characteristic absorption curve of visual purple. It is possible that the essential chemical change is one of *hydrolysis*."

Hitherto I have purposely omitted to mention the opinion of KÜHNE and EWALD which they expressed more than 70 years ago regarding the nature of the bleaching process: "Die einzige Ansicht, welche wir uns bis jetzt von dem photochemischen Zersetzungsprozesse des Sehpurpurs zu bilden vermögen, ist die, dass er in einer Wasserentziehung bestehe."

If the bleaching of visual purple is really caused by dehydration of rods, then the fluctuations of the retina's refractive index can be explained logically and simply in the following way:

The light adapted retina.

Through the action of the light visual purple is changed into indicator yellow by which a dehydration, exudation of water from the rods, takes place. When such a retinal preparation is pressed into the refractometer the anhydrous rods absorb water from the retina's other layers and the result is that the refractive index falls.

The dark adapted retina.

The retina contains visual purple, the rods are therefore water drenched and the refractive index lower than in the light adapted preparation. When the retina in the refractometer is illuminated the visual purple changes to indicator yellow. Water then disappears from the rods and the result is that the index rises. This rise, however, is of short duration and often low in degree: because the dehydrated rods soon regain their water equilibrium, that is to say, the original index value is restored when the rods from the neighbouring layers (with which they are pressed together) soon reabsorb the lost water.

If one takes into consideration how rapidly the visual purple solution bleaches in light as also how even the retina adapted to darkness hereby loses its reddish colour in a short time, one might possibly expect a greater rise in the refractive index if this rise really was an indicator for bleaching. Investigations, however, show that the *quantitative decomposition* of the visual purple does not take place so rapidly in the retina.

Thus ZEWEI came to the following conclusions during his experiments on frog's eyes: when he illuminated a dark adapted eye of a living experimental animal with a 20,000 meter-candle-power electric lamp, the quantity of visual purple fell by 50 % after half a minute's illumination. After the eye had been in this powerful light for 10 minutes, 20 % of the original visual purple was found to be remaining in the retina. In the *detached retina* the bleaching took place *more slowly*. If the dark adapted eye was illuminated by a 55 mcp lamp the quantity of visual purple fell during the first hour by 30 % only.

To the extent in which a frog's eye can be compared with the eye of a mammal, it is possible, with these experiments as a background, in the cases where the retinal refractive index remained constant during the whole time of observation, to imagine that the bleaching of the visual purple, that is its dehydration, and the absorption of water took place simultaneously and at the same rate. It is even possible, if also less probable, that both the rising and falling of the index value took place during the interval between readings. The readings in the 3rd and 6th rows of table 2 have obviously been obtained first after the index had risen by one decimal.

Even though the results obtained from dark adapted eyes are *inter alia* rather different, they all differ clearly and significantly from the results obtained from light adapted eyes. The index fall, so typical for the latter and which *always occurs* in them, does not occur in the first named.

Even though the dark adapted retinae give support, in so beautiful a manner, to KÜHNE's and EWALD's expressed supposition about a dehydration being the result of the illumination, the experiments carried out are, however, so few in number (20 in all) and the results so very varying that they have not got the same value from a point of view of proof as is the case with the index fall in the light adapted retinae. This fall could be established scores of times and, as I have already pointed out, it took place in every experiment.

Investigations concerning the dark adapted retinae ought therefore to be continued, with some other animal as an object, if one has not got a dark room at one's disposal where both adaptation and slaughtering can be carried out. In this case the cow is much too difficult to handle, and besides it even dangerous to life, so that adaptation to darkness was often unsuccessful because of the animal's nervousness and rage. When the "blind bandage" was removed in the dark room it was sometimes seen that it had become loose and that either one or both eyes were open. *In these unsuccessful tests it always happened that there was a regular index fall in the retinal sample.*

C. Light Adapted Eyes Examined after the First Hour post mortem.

In samples which were examined during the first hour post mortem, no correlation was discovered between the index value and the time which had elapsed after slaughter (Table 3). In the following we will examine the conditions prevailing with a preparation which is older than one hour, with especial regard to the occurrence of an index fall in these.

Table 1 shows the index values which were read every minute in retinae from $1\frac{1}{2}$ to $4\frac{1}{2}$ hours old.

We notice how the extent of the fall and its speed gradually decrease with time post mortem. In samples two hours old both are still equally large as those more often established in the age class "during the first hour post mortem", but in $2\frac{1}{2}$ hour-old and older samples the index does not fall more than one decimal. Further the reaction time begins to increase. The fall is just as great still in 4 hour-old retinae, but the reaction has now been delayed considerably. In $4\frac{1}{2}$ hour-old samples the index does not fall more during an observation time of 20 minutes. The index value is then already as low as 1,355.

Naturally the index fall is not always the same in samples of the same age. The idea with the exposition is merely to illustrate with a few examples the gradual decrease of the index fall, its slower course and its final dying out. Sundry eyes could show even greater deviations. The postmortal changes do not occur with the same speed in every retina.

The gradual fading out of the index fall depends apparently on the fact that the dehydrated rods already "in situ" gradually absorb water from the other layers of the retina. When such a

retinal sample is pressed between the prisms the index fall is already in process and only the final act of this fall takes place in the refractometer.

This absorption of water in the rods takes place much more slowly in unopened eyes than in the refractometer where the retina is exposed to pressure and where water is thus literally pressed around the anhydrous rods.

When the index has reached so low a value as 1,355 (the 4½ hour-old sample in Table 1), the rods are already saturated, so that no further index fall can take place. The low value speaks for the fact that postmortal processes such as autolysis which cause the water content of the tissues to increase, have already taken place. The index value thus reflects the water content prevailing at the moment. So, for instance, the refractive index in an eye which has been kept in a refrigerator for one day is usually approximately 1.344 and after 2 days 1.341.

It is easy to follow the advance of the postmortal changes from the appearance of the retina. In the region of Tapetum Lucidum especially, one can clearly see how this membrane, in fresh eyes from clear green to deep blue, gradually as if loses its beautiful colour in the same way that the retina becomes cloudy, swells and finally changes to a grey amorphous mass.

2. The Value of the Refractive Index.

A. Cows Eyes.

As already mentioned, the material comprises 321 retinal samples. The age of the animals was from 1 to 12 years and the examination of the preparations was carried out within the first hour post mortem.

Fig. 4 shows how the observations are classified into different index values. The diagram scarcely needs any explanation. In front of the rectangles, which represent the classification of the observations into different value groups, the normal frequency curve is drawn phantom-like.

The value obtained for the *refractive index in the retinae of cows* was 1.3610 ± 0.0008 .

In order to gain clarity regarding the eventual tendency of the index value to fall already during the course of the first hour post mortem, the material has been arranged into different time groups in relation to the carrying out of the examinations after slaughter. In this manner the following correlation table has been obtained.

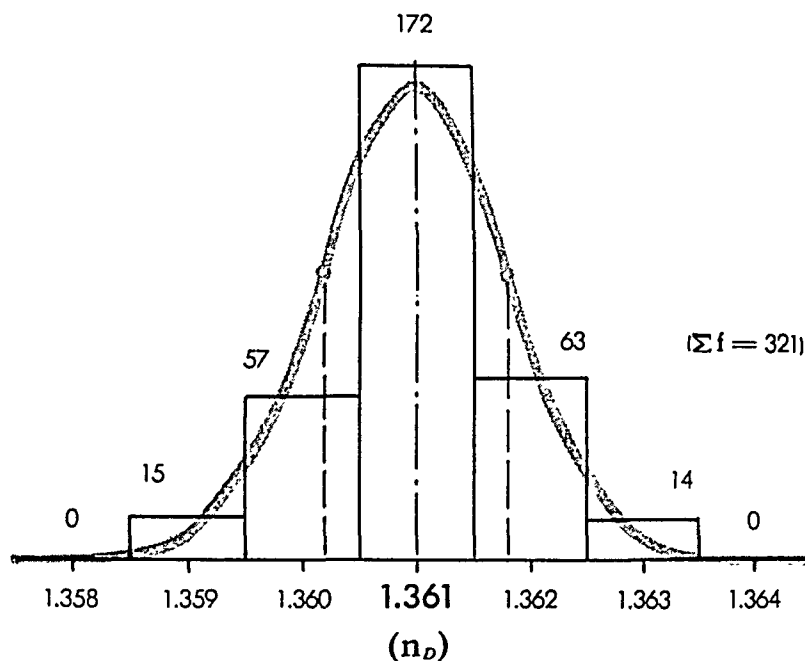


Fig. 4. Distribution of values of the refractive index (cows).

Table 3.

Correlation between the refractive index in cows eyes and the post mortem time elapsed before observations made.

t.	1.359	1.360	1.361	1.362	1.363	
5'....		5	10	3		18
10'....	2	5	8	7	1	23
15'....		5	13	5	3	26
20'....	3	7	14	7	4	35
25'....	2	5	17	1	1	26
30'....	2	5	24	7		38
35'....	3	8	20	8	2	41
40'....	2	6	26	6	2	42
45'....	1	8	17	7	1	34
50'....		2	13	9		24
55'....		1	10	3		14
	15	57	172	63	14	321

The calculated correlation coefficient r is:

$$r = \frac{S(xy)}{\sqrt{S(x^2) S(y^2)}} = \frac{26.54}{\sqrt{235.95 \times 2462.7}} = 0.034.$$

Thus no correlation between the age of the examined eyes and the refractive index value exists.

As the age of the animals varies so much, the question also arises as to whether age has any influence on the index values. The value of the correlation coefficient, calculated on the base of the following table, is:

$$r = \frac{-4.71}{\sqrt{235.95 \times 2142.5}} = -0.007.$$

Table 4.

Correlation between the refractive index and the age of the cows.

y.	1.359	1.360	1.361	1.362	1.363	
1		3	5	3		11
2		8	8	3	2	21
3	2	1	8	1		12
4		2	18	8		28
5	2	7	20	12	1	42
6		8	22	12	2	44
7	3	7	27	5	3	45
8	3	11	25	9		48
9	3	7	14	3	3	30
10	2	2	15	5	2	26
11			6	2	1	9
12		1	4			5
	15	57	172	63	14	321

Consequently the calculated coefficient points to the fact that the age of the experimental animal has no influence on the index value of the retina. The refractive indices of other tissues such as the liver, kidneys and so on (AURELL, according to a modification of Spalteholz's method) seem also to be independent of the age of the animal.

The sex of the experimental animal does not appear to influence the index value. When examining the eyes of bulls the index kept within the same borderline.

It seemed interesting to investigate whether such outward circumstances as nourishment, pregnancy, diseases suffered and so on affected the retinal refractive index in cows. Therefore the investigation was extended even to new-born calves.

It can be mentioned in this connection that amongst the opened eyes there was a case of traumatic cataract as well as a case of iridocyclitis purulenta. In both cases the index was 1.361, or just as great as in healthy eyes.

B. Calf Eyes.

The material comprises 104 retinal samples. The age of the animals varied from a few hours to one day at the most. All experimental animals were sucking calves. The material therefore was, with perhaps the exception of breed, rather homogenous.

The classification of the values of the refractive index appears in Fig. 5.

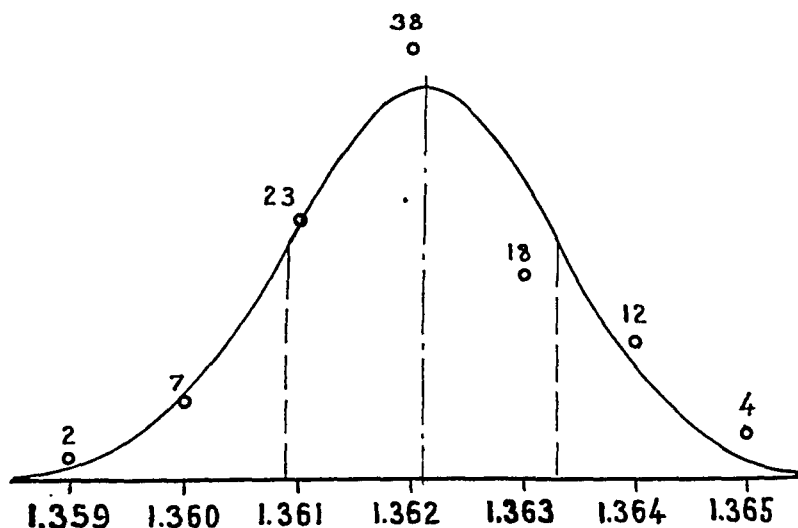


Fig. 5. Distribution of values of the refractive index (calves).

The value obtained for the *refractive index for the retinae of calves* was 1.3621 ± 0.0012 .

Both of these parameters are therefore greater than those obtained from the cow material. On account of this discrepancy as well as on account of the fact that we have previously arrived at the result that age does not influence the refractive index of the retina, we are now faced with the question as to whether the cow and calf material treated can be really said to belong to the same homology and that the difference depends solely on statistical reasons. The statistical method speaks for the contrary because the parameters in question differ from each other in so significant a manner.

This contradiction is, however, only seeming. The eyes of the cow material, regarding which the age of the animal proved to have no effect on the retinal refractive index, were fully developed but it can be supposed that the retinae in the calf material had not yet attained their final optical qualities especially as in this material one often came unexpectedly across lenses which, at this stage, were still partly in the process of development. In many of the calves the lenses were not yet fully transparent. (The material will be published later.) Under such circumstances as well as because the calf material is not included in the age interval from which the conclusions regarding the influence of age were drawn, there is, as a matter of fact, no contradiction between the observations made.

The discrepancy in the parameters depends partly on technical reasons connected with the preparation. Calf eyes are considerably more difficult to handle. Their size is smaller. All the membranes are thinner and more loose and, what is more important, more slimy which makes it more difficult to get a hold on them. These, for their part, cause a greater spreading of the value of the index of the material and, above all, the extremely rapid drying process influences the rise of the index value.

The index fall takes place in calves eyes with the same regularity as in cows' eyes and is approximately the same as in the latter though often even more rapid.

C. Pigs Eyes.

The eyes of pigs hitherto examined amount to 43 only, which, from a statistical point of view, is a comparatively small material.

The retina of a pig, however, differs from that of a cow or a calf in a rather considerable and interesting manner and is therefore worthy of mention in this connection.

In the first place the *refractive index is considerably lower*. The material examined shows a value of 1.35733 ± 0.00167 . Further the index fall of the light adapted retina in the refractometer is about 50 % lower than in cow's and calves' eyes. The index falls by 1—2 units of the third decimal only. In addition the speed of falling is often much slower. The ocular border line is more often quite unsharp.

What is the reason for these differences? Would it be too daring, on the basis of the hypothesis of dehydration of the rods, to expound the following: the retina of a pig is, so to say, a rod defi-

cient cone retina whilst that of a cow is, as known, rich in rods. For this reason therefore, the dehydration caused by light is less in the eye of a pig: that is to say, the initial value for the refractive index, read from the refractometer is lower, and the absorption of water, when the preparation lies between the prisms, is correspondingly less so that the index fall is even lower.

D. Horses Eyes.

So far, I have only examined the eyes of 12 horses.

The *refractive index* in 9 cases was 1.361 and in 3 cases 1.362. Index fall occurs regularly.

Summary.

1. As a rule the refractive index for dark adapted retinae of cows was 1.358.

2. In a material of 321 cases the light adapted retina's index was 1.3610 ± 0.0008 .

3. The experiments carried out support the supposition expressed by KÜHNE and EWALD that visual purple exudes water when it is bleached. For this reason the value of the refractive index in light adapted retinae is higher than that in dark adapted ones.

4. The retinal refractive index in newly born calves in a material of 104 samples was 1.3621 ± 0.0012 .

5. The refractive index for the retinae of pigs was 1.35733 ± 0.00167 . The low index value possibly depends on the fact that the retina is poor in rods.

6. The refractive index in horses' eyes was as a rule 1.361.

I have great pleasure in tendering my heartfelt thanks to Professor J. W. Nordenson on whose advice I began with the task of endeavouring to determine the refractive index of the retina. Thanks to the valuable and influential support which he gave me I was able to overcome all the difficulties which I encountered during the progress of the work and, in addition to this he gave me a great deal of invaluable advice. I wish also to thank Dr O. Brandt the Chief Veterinary Surgeon of the Stockholm Slaughter House for his never failing help which he so readily gave me during all the phases of my investigations. It was entirely due to him that I was able to collect so large a material under such favourable conditions. I also owe a great debt of gratitude to my dear brother Dr Reino Ajo for the advice which he gave me when working out the mathematical details of my work.

Last but not least, I wish to thank my beloved wife, Margareta Ajo, for all the encouragement and assistance which she gave me the whole time. The translation of my work into English was made by my old friend Captain Jack O'Brien-Hitching, M. C. to whom I give my best thanks.

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Experiments on the Influence of Electric Current Upon Growing Nerve Cell Processes in Vitro.

By

DAVID INGVAR.

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The morphogenetic factors at the outgrowth of nerve cell processes in vitro are mainly of a chemical, mechanical and electric character. WEISS showed (1929 and 1934) that mechanical factors are of great importance in the culture medium. Concerning the "chemotropism" investigations, also carried out by WEISS, show that it is not possible to prove this theory by experiments in vitro. The first attempt to find out the importance of electric factors was made by S. INGVAR (1920). He states that if a nerve cell culture is exposed to a weak electric current ($2 \cdot 10^{-12}$ — $4 \cdot 10^{-12}$ A) the nerve processes grow "along the lines of force in the galvanic field" and that if a conductor is laid through the culture the processes grow out at right angles to this.

In 11 cultures KARSEN and SAGER (1934) do not succeed in showing that the electric current has any certain influence on nerve processes in vitro. As electrodes they use "cathode-pulvérisation" which is spread out in 4 strings directly on the coverglass (hanging-drop cultures) so that the current can run in two directions perpendicular to each other. Tissue for the experiments is taken from cortex telencephali (embryons 11—14 days). The outgrowth obtained in the cultures is not at all uniform on all sides of the explantate. In one or two cases a syneresis in the plasma appears round the cultures which highly influences the mechanical factors in the medium. Only in some cases the authors seem to have obtained an outgrowth along the course of the cur-

For the performance of the experiments the author is indebted to Dr. Med. Albert Fischer and Professor Gösta Glimstedt.

rent but they regard this result as unreliable because of the uneven outgrowth on different sides of the explantate. They propose round cortex-pieces to avoid this. Against this one could object that cortex-cultures are not suited for such experiments as the neuroblasts here are already at an early stage well arranged and have a polarity that, in all probability, has an effect on the outgrowth of nerve processes. The explantates from telencephalon are also inconvenient considering the difficulty to avoid syneresis, which according to WEISS (1934) arises in connection with attached ependymal tissue.

In a more extensive paper WEISS (1934) tries to determine the factors which decide the direction of nerve cell processes in vitro. Among other things he tries to reconstruct S. INGVAR's experiments with nerve cell cultures exposed to electric currents. WEISS says that during one and a half year he tried more than 20 different types of methodes for the culture- and electrode-technique alone (intensity of current: 10^{-9} — 10^{-3} A; electrodes: chlorinated silver strings or mercury-calomel; tissue: telencephalon and spinal ganglions), without tracing any effect of the current. S. INGVAR's positive findings are claimed by WEISS to be a result of the suction that his electrodes made on the medium. For these consisted of pieces of cotton, of which one end touched the culture-medium and the other dipped down into a solution of a Zn-salt. By the suction, structures were made in the plasma which the growing nerve cell processes then followed. WEISS has shown the importance of such mechanical factors with great evidence, so there is good reason to suppose that the morphogenetic factors in S. INGVAR's experiments are mechanical and not electrical.

S. INGVAR explains the outgrowth perpendicular to a conductor passing in the medium as an effect of the vectors in the electric field of force round the conductor. He considers this to be an experimental parallel to the phenomenon first described by BOLK that the nerves grow out at right angles to the spinal cord. With our present knowledge of the electric phenomena which accompany the transmission in nerves, one will not be able in this way to compare the spinal cord to a conductor. The passage of the electric current through a conductor is certainly different in its essence from the transmission of the impulse in the nerve. WEISS is even in this case of the opinion that mechanical factors in the shape of tensions in the fibrin net round the conductor have decided the direction of the nerve cell processes in the plasma.

Material and Technique.

The ordinary culture technique was used. The medium consisted in one drop of plasma, one drop of Ringer and two drops of embryonic juice (II centrifugate). Nervous tissue was taken from chicken embryos which were 8—9 days old in all cases but three when they were 10, 12 and 13 days old. From these embryos lumbar ganglions were taken under sterile conditions and as much as possible of the root and nerve rests were cut away. The ganglions were used undivided to get the most uniform outgrowth round them.

To exclude the fibroblasts¹ which at the usual incubation temperature 38—39° C wander out in the medium at the same rate as the nerve processes grow out, a lower incubation temperature, 32° C, was used according to LEVI (1941). Hereby the nerve processes grew free from fibroblasts during the first two days. Not until the third day did the fibroblasts, repressed as they were in their growth and increasing, begin to reach out here and there on a level with the ends of the nerve processes.

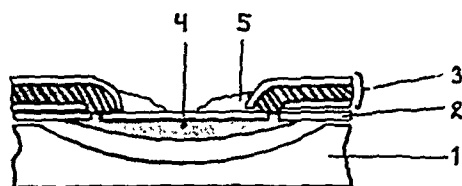


Figure showing culture and surroundings.

1. Hollow-ground glass.
2. Object-glass with two little holes.
3. Electrode-tube with sterile Ringer-agar.
4. The ganglion in the medium.
5. Paraffin.

Technique of culture and electrodes (See Fig.): The ganglion (4) was placed in the medium above-mentioned on an ordinary slide (2) for microscopical use. In this there were two little holes through which the current was conducted. The ganglion was placed right between these two holes. Here the density of current must be highest. During the application the slide rested on a sterile cover-glass so that the medium might fill out the holes. After the coagulation of the medium the hollow-ground glass (1) was fixed on the slide with paraffin. The sterile electrode tubes (3) (filled with sterile Ringer-agar) were then applied over the two holes and fixed there with adhesive tape. The gap between the electrode tubes and the object-glass was minimal as the tube had been ground even underneath. Care was taken that no air-bubbles which impaired the contact were enclosed in the holes

¹ The term fibroblast here comprises both cells of ectodermal and mesodermal origin which cannot easily be separated in vitro.

between the agar and the plasma. At last melted paraffin (5) was poured round the ends of the electrode tubes so that the evaporation might not increase the resistance. Through a hole in the paraffin the cultures could be observed and drawn in a microscope every day during the experiment.

The other ends of the electrode tubes dipped down into small vessels filled with Ringer in which chlorinated silver spirals hung down. The cultures were connected in series. The electric resistance in one culture was about 0.075 megohm. It increased twofold during the experiment partly due to polarization of the silver spirals.

The current circuit consisted in a) the cultures connected in series, b) microamperemeter, c) anode battery and d) adjustable resistances. Intensities of current from $2 \cdot 10^{-6}$ — $0.6 \cdot 10^{-3}$ A were used. In the first experiment the intensity was $2 \cdot 10^{-6}$ A and was then doubled at each experiment. The upper limit with this experiment technique turned out to be just above $0.6 \cdot 10^{-3}$ A. Already at $0.7 \cdot 10^{-3}$ A, the growth was slight and small gas bubbles appeared in the medium. If the intensity of current was further increased, the gas formation was also increased and the cultures shrunk. The cultures were regularly influenced during three days by the current which was kept fairly constant by the adjustable resistances.

Result.

Even with this technique the current had no influence on the direction of the outgrowing nerve cell processes. On the whole the outgrowth was uniform and radial round the ganglions and no orientation due to the current could be traced.

The outgrowth of control cultures was of the same kind. This suggests that with the technique used the structural changes in the plasma had been of so little importance that the outgrowth had not been influenced by it. The tail-like outgrowth from the remaining rests of the roots and nerves on the ganglion can be explained by the fact that here a certain amount of processes had the same direction at the beginning of the outgrowth. These tails too were uninfluenced by the current.

Discussion.

It can now be considered as a fact that cells and cell particles can possess potentials great enough to make them move in an electric field (SEIFRIZ 1939). This has been shown on nerve cells by PETERFI and WILLIAMS (1933). Then it would seem possible

that nerve cell processes growing in a medium that is passed by electric current would be influenced by electric forces.

LUND has succeeded (1924) in showing that stem internodes of the coelenterate *Obelia* placed in a small vessel with sea water can be influenced in their growth by electric current passing through the sea water. LUND shows that if the inherent potential difference between the apical and basal part of the internode is influenced in various ways by the current, differences will be produced in the size and direction of the outgrowth of the internode.

If there is a potential difference between the nerve cell body and the ends of its processes is not known to the author. If this was the case and if it was possible to measure such a difference it would be conceivable to analyse the influence of electric currents on nerve cells in vitro with a refined technique that mainly agreed with that of LUND's.

Summary.

With a new technique experiments are made to verify S. INGVAR's statement (1920) that electric currents can direct the nerve cell processes in vitro. Current intensities of 10^{-6} — 10^{-3} A were used. No influence could be traced.

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Some Perfusion Experiments on Horse's Brain.

By

P. ASTRUP, G. STEENSHOLT and K. WAMBERG.

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Introduction.

The metabolism of brain tissue, or of nervous tissue in general, is, in spite of the great amount of work expended on the problem, still very obscure in several important respects. Most investigations on this problem have been carried out by means of the BARCROFT-WARBURG technique (cf. QUASTEL (1939)), and valuable information has been obtained in this way concerning enzyme systems, in particular those catalysing oxidative processes. The desirability of supplementing these investigations by experiments *in vivo* has certainly been clear to many workers in this field, but so far only a few perfusion experiments have been reported in the literature. It must be admitted that the results hitherto reported have been rather meagre. The first investigations of this kind were carried out by comparing the cerebral arterial and venous blood. This method suffers from the disadvantage that the blood flow through the brain could not be controlled and measured with sufficient accuracy on account of the complicated blood supply to this organ. It should be noted, however, that recently developed methods may permit great improvements to be made in the measurement of blood flow in intact animals (see for instance KETY and SCHMIDT (1945)). Another disadvantage of the procedure is the fact that most of our present analytical methods are hardly adequate to deal with such small differences in composition as those between cerebral arterial and venous blood. It seems that only problems of gaseous metabolism can be approached by

this method with any reasonable hope of success. Recourse must therefore be had to methods based on direct perfusion of the brain, where the blood flow is under control during the whole experiment. Only very few investigations of this kind have been carried out so far. Thus SCHMIDT (1928) and HEYMANS and BOUCKAERT (1935) have described perfusion methods, but these authors were not primarily concerned with studies of brain metabolism. From the literature available to us it appears that the only other investigations in this field are those of CHUTE and SMYTH (1939), of SCHMIDT, KETY and PENNES (1945), and of HANDLEY, SWEENEY, SCHERMAN and SEVERANCE (1943). These authors worked with cats, monkeys and dogs respectively. Their results will be considered later in this paper. We would like to mention, however, that the work of the American groups became known to us only some time after the war and after the completion of the experiments reported in the present paper.

Our own work originated independently of all previous investigations in this field. A justification for the publication of the present report may be seen in the fact that, in view of the great difficulties inherent in the problem, we are in need of experiments carried out on different animals and by different procedures.

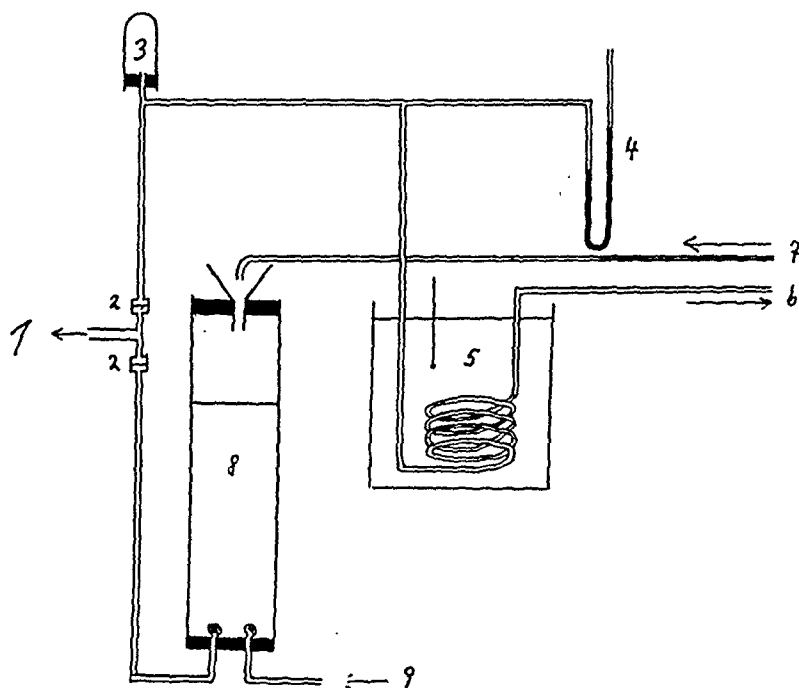
Experimental Part.

Perfusion technique. All our experiments were carried out on horse's brain, mainly because we found it technically more convenient to work with organs of this size, rather than with the small brains of cats and dogs. Thus, the total amount of blood in the perfusion system is so large that many samples can be removed for analysis without appreciably altering the amount of blood.

Our apparatus was of a type familiar in perfusion experiments (see TIGERSTEDT (1911)), and was in all essentials similar to that employed by for instance NIELSEN (1932) and BLIXENCRONE (1938). It is shown diagrammatically in the accompanying figure.

In all experiments we used heparinized blood, drawn from the animal before the operation and before the narcotization. The oxygenation was carried out by means of a mixture of 95 % oxygen and 5 % carbon dioxide.

The horses used in the experiments had been bought by Den Kgl. Veterinær- og Landbohøjskole for purposes of instruction in the surgical and anatomical departments. Before our perfusion experiments they had been used in the surgical department by the students; and had been anesthetized for 2—3 hours with chlorale hydrate. The operations carried out were of a type commonly met with in veterinary



- 1: Pump.
- 2: Valves.
- 3: Buffer.
- 4: Manometer.
- 5: Water bath.
- 6: To the brain.
- 7: From the brain.
- 8: Oxygenator and blood reservoir.
- 9: Oxygen supply.

practice. They were of a more peripheral nature, and none of the larger organs were touched. Our experiments started immediately after these operations had been finished.

In order to isolate the brain and connect it to the perfusion apparatus we adopted the following procedure: We ligated, on one side of the neck, arteria carotis communis and arteria carotis externa and interna as well as arteria occipitalis, arteria thyroidea cranialis and arteria pharyngea ascendens. Finally vena jugularis and vena maxillaris interna and externa were ligated. Then, on the other side of the neck, we isolated arteria carotis communis, and ligated arteria carotis externa, arteria occipitalis, arteria thyroidea cranialis and arteria pharyngea ascendens. We also ligated vena maxillaris externa. A cannula was then placed in vena jugularis and the blood allowed to flow down into a recipient. The blood supply to the brain was now stopped for a short while by a clamp on arteria carotis communis, and a cannula already connected to the perfusion apparatus was placed in this artery above the clamp. The perfusion was now started. Immediately afterwards the animal was killed by cutting aorta.

Methods of analysis. The gas analyses were carried out by the standard methods described in PETERS and VAN SLYKE (1932).

The determinations of glucose were made by the HAGEDORN-NORMAN JENSEN method.

Cholesterol was determined by the method of SCHÖNHEIMER and SPERRY in the modification worked out by BRUN (1939). The fat analyses were also carried out by familiar methods. We used a procedure described by BRUN (1939).

In the horse there is, as in man, a *circulus arteriosus* (Willisi). The blood supply to this and consequently also to the brain, is mainly through *arteria carotis interna*, and only to a small extent through *arteria vertebralis*. In all perfusion experiments it is essential to get a closed system, with no loss of perfusion fluid. In the case under consideration loss of blood can take place through arterial anastomoses between arteries in the brain and *arteria vertebralis*, and the artery passing along *medulla spinalis*. We have tried to avoid this loss of blood by ligation of *arteria vertebralis*, but without much success. In our experiments, a certain small fraction (ca. 5 %) of the fluid always disappeared from the perfusion system. This fraction remained remarkably constant during the experiments. In some preliminary experiments we tried to find out by which passages this blood disappeared, for instance by separating the head of the animal from the body. We came to the conclusion that the blood disappeared by arterial anastomoses leading to the artery passing along *medulla spinalis*, and we consider it probable that there was no loss of venous blood.

These conclusions were substantiated by an experiment in which black ink was added to the perfusion fluid. Subsequent dissection of the head of the animal showed that practically only the brain was perfused, and the other tissues which were coloured by the ink (*palatum durum* and part of the cartilage in the nose) can hardly be supposed to make appreciable contributions to the metabolic changes in the blood.

An exhaustive report of all experiments cannot be given for reasons of economy of space. We shall therefore limit ourselves to the description of a typical experiment chosen at random from the laboratory journal.

2,100 ml of blood were drawn from the *vena jugularis* 3 hours before the experiment and before the animal was chloralized. 330 mg heparin were added. Shortly before the experiment was started the blood was put into the perfusion apparatus. The temperature of the blood on leaving the arterial cannula was 37° C. The experiment was started as described above, and the apparatus adjusted to give a uniform blood supply of 230 ml per minute. The experiment was allowed to run 10 minutes before the first blood samples were removed for analysis of gas, glucose, rest nitrogen, cholesterol and fat. The next samples were drawn after 30 minutes and 60 minutes respectively. During the perfusion the venous outflow

was measured every fifth minute and remained constant at 210 to 220 ml per minute. The blood pressure was about 100 mm Hg.

In work of this kind it is clearly desirable to have some indications of the persistence of cerebral activity during the experiments. In the present case the corneal reflex persisted for 40 minutes, and during the first 20 minutes there were rhythmical respiratory movements of the nostrils. Movements of the ears and spontaneous movements of the eye-lids were also observed.

The hematocrite values decreased during the experiment from 25 to 23.

The weight of the brain was 550 grams.

The results of the analyses were as follows:

The oxygen consumption was found to be 190 ml per 100 g brain tissue per hour, with a respiratory quotient of 0.88. We further found a consumption of glucose amounting to 150 mg per 100 gr tissue per hour. The blood cholesterol and blood non-protein nitrogen both remained constant during the perfusion. We finally observed a decrease in the total blood fat of 100 mg per 100 gr tissue per hour.

All in all 14 experiments of this kind were carried out. The respiratory quotient was always found to lie between 0.88 and 0.99 while the oxygen consumption varied from 146 to 300 ml per 100 gr tissue per hour. In two of our experiments the blood fat remained constant, in the others a decrease was observed ranging from 100 mg to 375 mg per 100 gr tissue per hour. The glucose consumption was always found in the range 125 mg to 260 mg per 100 gr tissue per hour. In all our experiments the non-protein nitrogen was practically constant during the perfusion, as was also the blood cholesterol. The blood pressure ranged from 60 to 120 mm Hg.

Discussion.

The values given above for the oxygen consumption are in fair agreement with those of other workers in this field. CHUTE and SMYTH (1939) and likewise SCHMIDT, KETY and PENNES (1945) gave results lying in the same range as ours; HANDLEY et al. (1943), however, found in their experiments on dogs an oxygen consumption as high as 546 to 796 ml per 100 gr tissue per hour. All workers agree with respect to the values for the respiratory quotient. In our experiments the blood sugar was always found to decrease, and our actual values for the consumption of glucose

agree well with those of previous writers, although our results seem to point to a somewhat lower glucose consumption in horse's brain than that found by CHUTE and SMYTH (1939) for cat's brain (150 to 400 mg glucose per 10 gr tissue per hour). The conclusion therefore seems justified that all these physiological quantities are now known with reasonable accuracy.

The results obtained for cholesterol and non-protein nitrogen do not seem to call for much comment at the present time.

A curious feature of most of our experiments was the disappearance of fat from the perfusion fluid. Unfortunately we are unable to offer any definite explanation for this. The high values of the respiratory quotient show clearly that at best only a very small fraction of the fat can have been oxidized. Moreover, the prevailing view, supported by all previous investigations in this field and certainly not contradicted by ours, is that brain cells consume only glucose. For some other types of nervous tissue, however, the case is perhaps different; thus, peripheral nervous tissue is said to have a respiratory quotient around 0.7.

In concluding this report some critical remarks on our experimental procedure may not be out of place. The technique of brain perfusion is difficult and it is not easy to comply rigorously with all requirements of stable experimental conditions. Thus, as already mentioned, we encountered difficulties in keeping the volume of the blood in the perfusion apparatus absolutely constant during the experiment. This is probably one of the main sources of error in our work. Account has been taken of this circumstance in the calculation of the numerical results. We may further mention the question whether the blood supply to the brain has been entirely adequate. The rate of perfusion was in our experiments 40 to 50 ml per 100 gr tissue per minute. CHUTE and SMYTH (1939) used 60 to 90 ml, SCHMIDT, KETY and PENNES (1945) used on an average 47 ml and HANDLEY et al. (1943) used 200 to 300 ml per 100 gr tissue per minute. These values are rather discordant, and divergent opinions seem to be entertained on this question by workers in this field. We hope at some later date to present further results pertaining to all these questions.

Summary.

The paper gives a report on some perfusion experiments on horse's brain. Results are given for the consumption of oxygen

and glucose and for the respiratory quotient. Some anomalies and technical difficulties encountered in the experiments are briefly discussed.

The authors are glad to express their thanks to P. Carl Petersens Fond, for generous financial support of our work. We are likewise greatly indebted to Dr. E. JORPES, Stockholm, and to Løvens Kemiske Fabrik, Copenhagen, for valuable gifts of heparin.

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On the Mechanism of Reciprocal Innervation.

By

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An afferent volley produced by a single shock applied to the sciatic nerve elicits in the decerebrated cat periodic reciprocal (SHERRINGTON) activity in the

extensors and flexors of the limbs. Activity in the extensors coincides with inactivity in the flexors. Fig. 1 A shows such activity recorded from the biceps and triceps muscles of the fore limb (moderate stretch) after stim. of the ipsilateral sciatic. If the local proprioceptive background is changed by stretching the extensor the periods of extensor activity lengthen while the active phases of the flexor shorten until they ultimately disappear. The last remnants of the periodic discharges in the flexor are found located between the

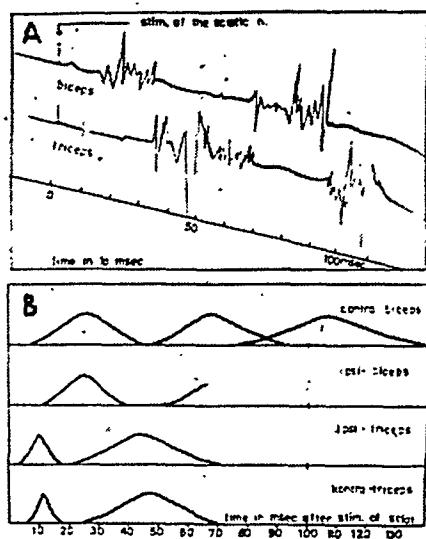


Fig. 1.
See text.

heavily extended extensor periods. Fig. 1 B is a diagrammatic representation of these phenomena illustrating the time relations of flexor and extensor activity in the fore limbs of both sides. The top of each curve represents the moments at which the last vestige of the process disappears in response to variations of the proprioceptive background. *Thus the afferent volley evokes a*

periodic activity in the flexor and extensor systems of regular and fixed time relation.

The cyclic changes in the two systems can be more accurately evaluated if the afferent stimulus is weak and the muscles recorded from are stretched but slightly, so that the action potentials can be recorded in the shape of discrete spikes (units or synchronized units). The reflex spikes then tend to group themselves within fixed periods after the shock. Figs. 2 C and D, based on several repetitions of the procedure, show in each column the number of muscle spikes per unit time in the left m. gastroc. and m. tib. ant. following stimulation of the contralateral (right) sciatic. The number of spikes is expressed in per cent of each maximum. Simultaneous records were taken of the slow potential changes in the ipsilateral (left) ventral root L7. One electrode was placed on the intact root just outside the spinal cord, the other electrode on

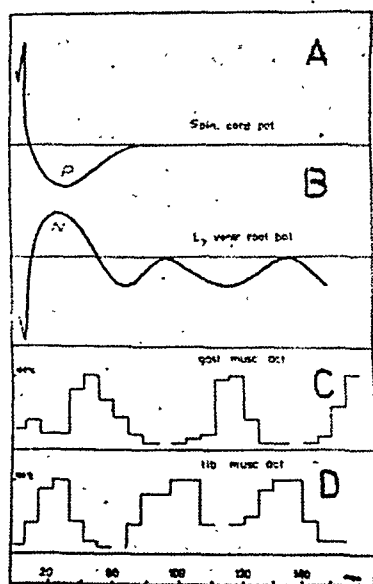


Fig. 2.
See text.

the bone of a spinous process. The time course of the polyphasic slow potential recorded in this manner is found in Fig. 2 B, adjusted to the same time axis. It is seen that there is a *fixed relationship between the positive and negative phases of the root potential* (root negativity upwards) *and the discharge in flexors and extensors*. Fig. 2 A illustrates the cord potential (cf. GASSER and GRAHAM, HUGHES and GASSER) in the same cat, recorded from the dorsum of the cord. The first negative phase N of the ventral root potential obviously corresponds to the positive phase P of the cord potential.

Does the reflex facilitation of respectively flexor and extensor activity correspond with opposite electrical sign of the slow ventral root potentials? In order to investigate this point two methods of approach have been used: (i) records have been taken of *the slow potential changes in the ventral root* (Fig. 3 B and F) with leads near the spinal cord and at the cut end (cf. BARRON,

and MATTHEWS, ECCLES) a) after multisynaptic flexor motor fibre activity (stimulation of the sural nerve, cf. LLOYD) and b) after monosynaptic extensor motor fibre activity (stimulation of the gastrocnemial nerves, cf. LLOYD); (ii) *the late excitability changes* following upon the reflex discharges in the two systems have been measured (Fig. 3, C, D, E).

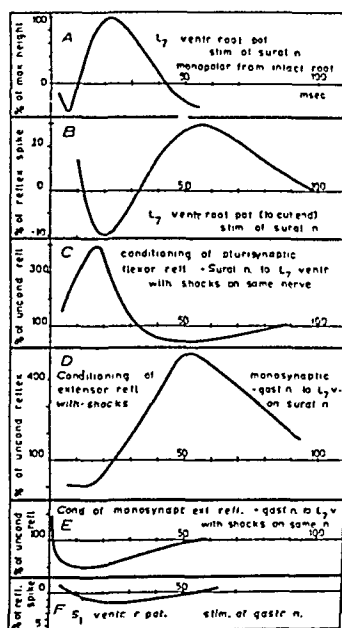


Fig. 3.
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but is clearly *subnormal under the later negative phase of the root potential*. The excitability curve for the extensor reflex in 3 D behaves in an exactly opposite manner. *The extensor reflex is wholly absent during the positive phase and enormously facilitated during the later negative phase (500 %) in record 3 B*. There is no such facilitation of the extensor reflex from the gastrocnemial nerve if the same nerve is conditioned (3 E), *i. e.* in the monosynaptic reflex. This is not either followed by a late root negativity (3 F). If anything, there is rather a small root positivity in 3 F. During this time the extensor reflex is subnormal (3 E) and this subnormality should be compared with the subnormality in 3 D corresponding to the root positivity in 3 B.

In view of the observations recorded above it was of great interest to study the afterpotentials in the gastrocnemial and tibial nerves. The sequence of potential changes as well as the excitability changes were therefore measured in excised nerves

of previously deafferented or deafferented cats. As representatives of flexor or extensor function the nerve branches to the gastrocnemial and tibial muscles were used for recording and stimulation.

The general characteristics of afterpotentials were found in both motor and sensory nerves of the flexor-extensor system. However, the negative and positive after potentials were greatly variable and unstable.

When these were simultaneously recorded in flexor and extensor nerves (Fig. 4 and 5) direct comparison seemed possible. In motor nerves some differences were found with respect to the positive afterpotential, whereas the simultaneously recorded negative afterpotentials were

found to be of equal size and shape. Thus, for instance, at pH 7.3, in the tibial muscle-nerve the positive wave was usually lacking or very small (cf. 4 A and 4 E). Under similar conditions in *pure sensory nerves* (5, A₁ and E₁) the positive waves were seen to be equal in the two types of nerve while the negative afterpotential of the gastrocnemial nerve was smaller than that of the tibial nerve. In a general way the afterpotentials, for certain stimulus strengths, were reflected by corresponding excitability changes (Fig. 4, B, C and 5; Fig. 5, C, G). When the nerve was tested with a train of shocks a characteristic rhythmic variation of excitability was noted. These alternating supernormal and subnormal phases usually occurred at slightly different rates in the two types of fibre, somewhat faster in the tibial nerve. Similar differences in the rhythmic changes of the excitability were also seen during unconditioned tetanic stimulation.

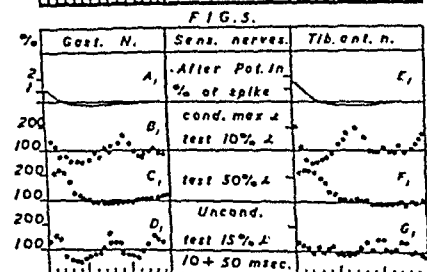
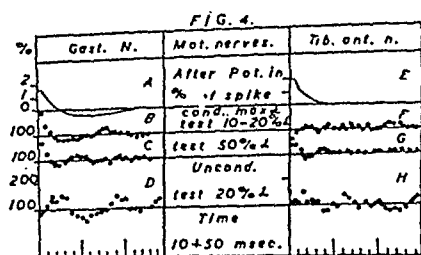


Fig. 4 and 5.

Afterpotential and excitability changes of motor (Fig. 4) and sensory nerves (Fig. 5). Excised and soaked 30–60 min. in Krebs solution, saturated with 95 % O₂ and 5 % CO₂; 37° C. Simultaneous recording of action pot. in gastr. and tib. muscle nerves; interelectrode distance 17 mm. Conditioning and test shocks through different pairs of electrodes on the sciatic nerve. Train of test shocks delivered at a tetan. freq. of about 200 c. p. s. Conduction dist. in Fig. 4 A and E 23 mm, in Fig. 5 A₁ and E₁ 30 mm. Motor or sensory roots cut (L₅–S₁) 3–4 weeks previous to experiment.

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On the Effect of Adenosine Triphosphate on Myosin Threads.

By

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The use of myosin as a model for certain properties of the muscular substance originates from investigations of v. MURALT and EDSALL (1930) and WEBER (1934). Following the discovery of ENGELHARDT and LJUBIMOVA (1939) on the adenosine triphosphatase activity associated with myosin, NEEDHAM et al. (1941) have analysed the changes in the configuration of the myosin molecule produced in myosin solutions by adenosine triphosphate (ATP). SZENT-GYÖRGYI and his school (1945) made extensive studies on the physical and chemical properties of muscle protein. They found that the contractile protein (actomyosin) is a compound of two components, actin and myosin, and demonstrated a striking effect of ATP on threads drawn from this substance, viz. an extreme volume constriction, used as a basic model for muscular contraction.

In recent investigations it has been found (BUCHTHAL et al. 1944, 1946) that ATP and some related compounds in minute amounts release contraction and change birefringence in the isolated muscle fibre. In view of these remarkable effects on muscle under physiological conditions and on myosin threads, it seemed of importance to investigate, how far the changes pro-

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duced by ATP in model experiments are related to the effect of this substance in muscle.

Method.

Preparation of myosin threads.

The myosin was extracted from rabbit muscle by the method of BANGA and SZENT-GYÖRGYI (1941) with certain modifications and corresponds probably to their myosin B or acto-myosin. Rabbits (after 24 hours fasting) were killed by stunning and packed in ice for 15–30 minutes. The muscles of the hind limbs and the back were excised and minced at a temperature below 5° C, and extracted for three days at 0° C, with three times their volume of Weber's solution (0.6 M KCl, 0.01 M Na₂CO₃ and 0.04 M NaHCO₃ per liter). After dilution of the extract with the same volume of Weber's solution and centrifuging (or filtering through gauze) the myosin was precipitated by adding 6–8 volumes of distilled water to 1 volume of the crude extract. After standing 24 hours at 0° C the solution is decanted and the precipitate centrifuged. The precipitate is washed with distilled water and brought in solution again by adding enough solid KCl to make the resulting solution 0.5 molar. If not quite clear, the viscous solution is centrifuged, thereby removing any insoluble protein. Three such precipitations were carried out.

Threads prepared from such a myosin, containing adenosine triphosphatase retain their enzyme activity over a long period. Myosin threads four months after preparation still had the original activity.

In order to obtain threads free from adenosine triphosphatase the precipitation of the crude myosin extract was carried out by dialysis against 0.005 M veronal-HCl buffer of pH 6, following the directions of SINGER and MEISTER (1945). After three precipitations by dialysis, a myosin practically free from adenosine-triphosphatase activity was obtained. The determination of the enzymatic activity of myosin solutions was carried out according to BAILEY (1942). The same procedure was applied to threads.

The threads were prepared by pressing out the myosin solution from a fine pipette into distilled water contained in a slowly rotating shallow glass vessel. They were generally kept in distilled water for 1–2 hours before use, and can be thus stored for several days at 0° C. Besides these "fresh threads" we have used threads prepared by drying the fresh thread in air for 20–30 minutes and imbibing it with 0.9 per cent NaCl solution for 30 minutes before use. Threads which are kept dry for more than a week do not show a shortening under the influence of ATP.

The analysis of a standard sample of our myosin solution used for the preparation of threads gave a dry weight of 3.2 per cent and the dried thread had a nitrogen content of 16.15 per cent.

The volume changes produced by ATP were determined on enlarged microphotographs of myosin threads, 3–5 mm long, or in some cases

by direct measurements under the microscope using an eye piece micrometer for measuring the length and the diameter of the thread. In these experiments the thread was kept on a celluloid slide in 0.9 per cent NaCl solution at pH 7.3. Glass slides proved to be less suitable, the threads having a tendency to stick to glass, whereby shortening may be hindered. Observations were carried out for a period of at least 20 minutes at 18–19° C.

Birefringence was measured with a BABINET compensator in white light and the diameter of the threads was determined by means of an eye piece micrometer with movable cobweb. When birefringence was examined in threads at constant length the arrangement described in a previous communication was used which allows measurements of phase difference and the thread diameter in the same plane (BUCHTHAL et al. 1946).

Determination of tension, length and dynamic elasticity.

Determinations of dynamic stiffness $\left(\frac{\Delta \text{ tension}}{\Delta \text{ length}} \right)$ were performed under isometric conditions on non-contractile threads (kept dry for more than a week) by continuous measurement of the tension, which is produced by small periodic variations in length of a frequency of 100 cycles per sec. The same technique was applied to isolated muscle fibres and is described in detail in a former publication (BUCHTHAL, KAISER and KNAPPEIS 1944).

For the determination of stiffness and tension in the contractile threads we used a different arrangement which allows the measurement of small periodic changes in length in a thread under constant load. For this purpose the thread is fastened in a pair of microtweezers which are soldered to a lever. The lever is in rigid connection with the moving coil of a galvanometer. The direct current which passes through the moving coil is thus proportional to the stress acting on the fibre. The direct current is superposed by a weak alternating current from a low frequency laboratory generator (frequency 5–20 cycles per sec.). Thereby tension varies periodically and induces changes in length which are inversely proportional to the stiffness. The changes in length are measured by optically registering the excursions of a mirror on the moving coil. By simultaneous records of the current in the moving coil and the deflections of the mirror it is possible to determine the phase difference which might occur between the force exerted on the fibre and the length alterations, when allowance is made for the mechanical constants of the moving system. This phase difference is a gauge for the viscous component of stiffness in the myosin thread at the respective measuring frequency.

Substances applied.

Adenosine triphosphate, adenosine diphosphate, inosine triphosphate and creatine phosphate were prepared as described previously (BUCHTHAL et al. 1946) and applied iso-osmotically as the sodium salts at pH 7.3.

Sodium triphosphate, sodium pyrophosphate and sodium orthophosphate were used as described in a previous communication (BUCHTHAL et al. 1944).

Acetylcholine chloride (Roche) was used in an iso-osmotic NaCl solution containing 100 μg acetylcholine per ml.

Porphyrexid was applied in the form of 0.0005 and 0.001 molar solutions in 0.9 per cent NaCl. The solutions were freshly prepared before use.

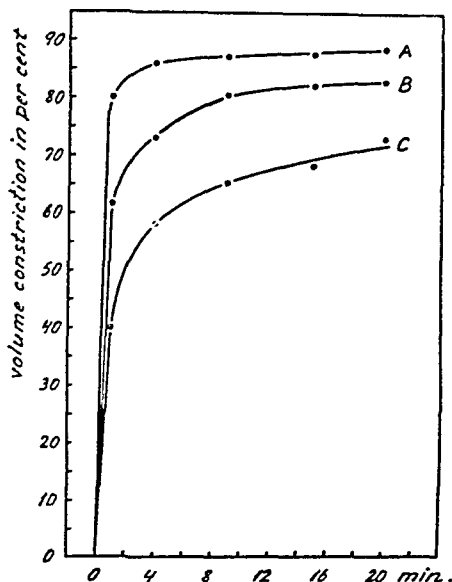


Fig. 1. Volume constriction of freshly prepared myosin threads at different concentrations of ATP.

Curve A: 7.2×10^{-5} mol/ml.

Curve B: 2.4×10^{-5} mol/ml.

Curve C: 1.2×10^{-5} mol/ml.

Abscissa: Time in minutes.

Ordinate: Volume constriction in per cent.

produced by ATP are highly specific, equimolar concentrations of inosine triphosphate, adenosine diphosphate and inorganic triphosphate being ineffective. Its effect on myosin threads is independent of the way the myosin has been prepared. Myosin purified according to SINGHER and MEISTER (1945) showing only slight ATP-ase activity gives the same decrease in volume as threads made from myosin prepared by other methods. ATP applied to dried myosin threads which have been immersed in an iso-osmotic NaCl solution before use, produces a decrease in length which, however, is accompanied by an increase in diameter, so that the volume of the threads remains unchanged.

Treatment of fresh threads with sulphydryl reagents *i. e.* mono-

Results.

Application of ATP in concentrations of 2×10^{-5} mol/ml causes shrinking of the myosin threads as already observed by SZENT-GYÖRGYI et al. (1941). The volume constriction as function of time at different concentrations of ATP is shown in Fig. 1. The relative shortening decreases with increasing initial diameter of the thread. In agreement with SZENT-GYÖRGYI et al. we find that the effect of ATP is enhanced in a solution containing 0.01 mol magnesium and 0.1 mol potassium per liter. The changes pro-

iodoacetic acid and porphyrexid (0.0005 molar) causes a considerable decrease in the volume constriction at subsequent application of ATP, porphyrexid practically inhibiting all volume changes at a concentration of 0.001 molarity (Fig. 2). Potassium ferricyanide is, however, without effect.

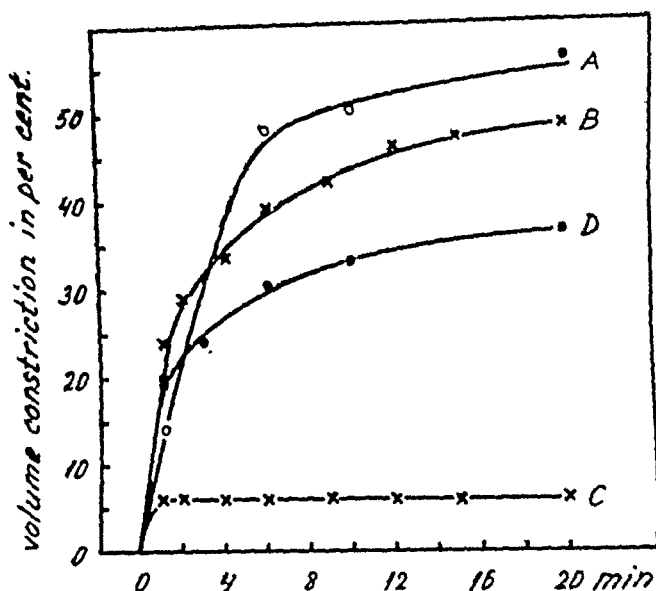


Fig. 2. Volume constriction of freshly prepared myosin threads caused by ATP (2×10^{-6} mol/ml) after previous treatment with sulfhydryl reagents.

Curve A: ATP.

Curve B: ATP after treatment with 0.0005 molar porphyrexid.

Curve C: ATP after treatment with 0.001 molar porphyrexid.

Curve D: ATP after treatment with 0.0005 molar monoiodoacetic acid.

Abscissa: Time in minutes.

Ordinate: Volume constriction in per cent.

Triphosphate, pyrophosphate and ortophosphate inactive as such in concentrations of 2×10^{-6} mol/ml, likewise reduce the effect of subsequently applied ATP, the inhibition being most pronounced with triphosphate and decreasing with a decreasing number of phosphate groups in the molecule (Fig. 3). On the other hand, inosine triphosphate (2×10^{-6} mol/ml) enhances the volume constriction produced by subsequent doses of ATP by approximately 10 per cent. Creatine phosphate (concentration 2×10^{-6} mol/ml) or creatine phosphate + adenylic acid in the same concentrations have no shortening effect on the myosin thread. While creatine phosphate reduces the effect of subsequently applied ATP approximately 15 per cent, adenylic acid alone or with

creatine phosphate does not alter the volume constriction normally produced by ATP.

Freshly prepared myosin threads which are kept in a 0.9 per cent NaCl solution can be stretched to thrice their initial length. The birefringence at equilibrium length is too low to be useful

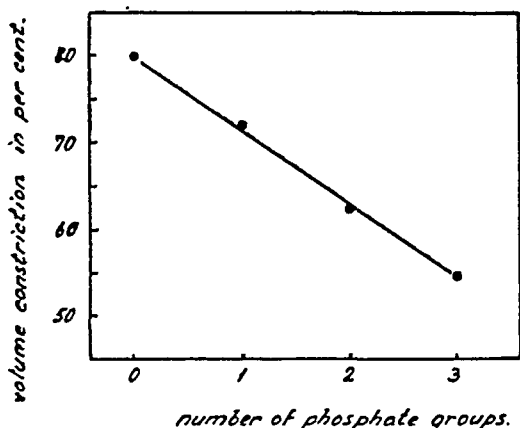


Fig. 3. Volume constriction of freshly prepared myosin threads caused by ATP (2×10^{-6} mol/ml) after previous treatment with sodium ortophosphate, sodium pyrophosphate and sodium triphosphate (2×10^{-6} mol/ml).

Abcissa: Number of phosphate groups per molecule.

Ordinate: Volume constriction in per cent.

for a study of changes induced by the substances in question. It increases, however, considerably with stretch (tension) from about 0.02×10^{-3} at equilibrium length (length 100) to 2.0×10^{-3} at length 275. By drying the threads in air, birefringence is increased from about 0.02×10^{-3} (wet thread) to 1.25×10^{-3} (dry thread). Volume constriction produced by ATP decreases the more, the longer the thread had been kept dry before re-immersing in 0.9 per cent NaCl, independent of

whether drying took place in air or in a nitrogen atmosphere. Thus it is evident that the orientation produced by drying is accompanied by other changes in the physical properties of the protein, probably a partial denaturation. The effect of ATP on myosin threads disappears completely after a certain time of drying which varies for different preparations from several hours to about a week.

Of the substances investigated only ATP causes a change in birefringence. This change can only be observed in the previously dried thread, which is re-immersed in a sodium chloride solution and treated with different concentrations of ATP. In a thread which is allowed to shorten without load, birefringence decreases after application of 2×10^{-6} mol/ml ATP by approximately 40 per cent (Fig. 4). Like volume constriction, this decrease is reduced by sulfhydryl reagents, creatine phosphate and by the inorganic phosphorous compounds investigated. The inhibitory effect

of porphyrexid on both birefringence and shortening is greater in dried than in freshly prepared threads. When shortening of the dried threads is prevented, ATP causes an increase in birefringence, while the diameter remains unchanged when a concentration of 2×10^{-6} mol/ml is applied (Fig. 7 B). With lower or higher concentrations the threads swell and values of birefringence are difficult to estimate. We did not succeed in counteracting this swelling effect by immersing the myosin thread in hypertonic solutions.

In freshly prepared myosin threads treatment with acetylcholine (100 μ g/ml) has no effect on the volume constriction caused by subsequent application of ATP. Identical results are obtained both with threads from enzymatically highly active myosin and from myosin with only slight activity. However, in "dried" myosin threads of low enzymatic activity, previous treatment with acetylcholine reduces the shortening to about one half.

On the other hand, in threads prepared from an enzymatically active myosin the shortening is unaffected by previous treatment with acetylcholine.

In enzymatically active myosin threads, which are immersed in an ATP solution ENGELHARDT et al. found an increase in extensibility of 50–100 per cent. This effect is specific for ATP and assumed to be present as long as enzymatic activity persists. The myosin threads applied were prepared according to WEBER (1934) and apparently did not show a volume constriction. In enzymatically active, non-contractile threads we have likewise found this increase in extensibility in measurements of the static and dynamic elastic properties. In the myosin threads, as in muscle,

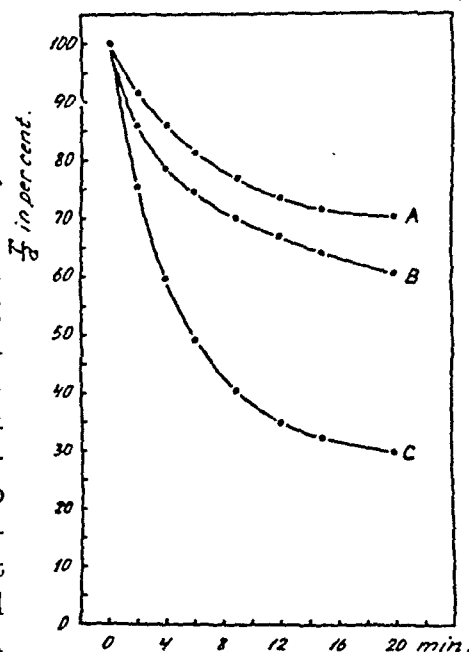


Fig. 4. Changes in birefringence of dried myosin threads (tensionless) at different concentrations of ATP.

Curve A: 1×10^{-6} mol/ml.

Curve B: 2×10^{-6} mol/ml.

Curve C: 4×10^{-6} mol/ml.

Abscissa: $\frac{l}{l_0}$ in per cent.

Ordinate: Time in minutes.

stiffness $\left(\frac{\Delta \text{ tension}}{\Delta \text{ length}}\right)$ increases proportionally with tension. When a myosin thread is immersed in ATP, the stiffness-tension diagram

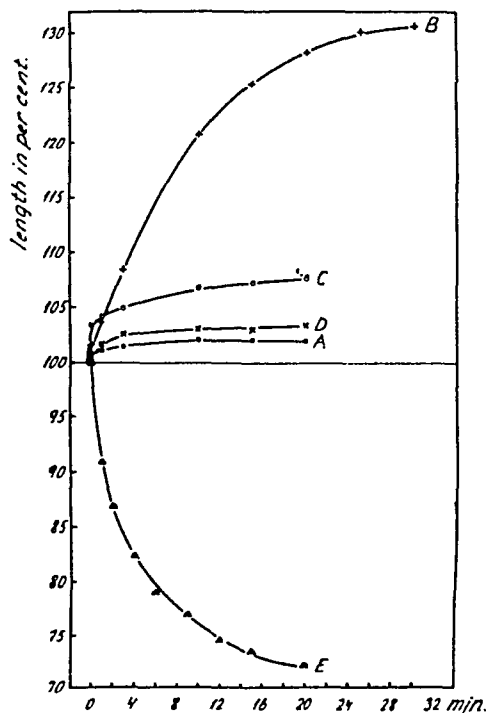


Fig. 5. Changes in length of dried myosin threads produced by ATP (2×10^{-6} mol/ml) at different loads.

Curve A: Untreated myosin thread, load 180 mg.

Curve B: Myosin thread after application of ATP, load 110 mg.

Curve C: Myosin thread after application of ATP, load 20 mg.

Curve D: Myosin thread after application of ATP, load 5 mg.

Curve E: Myosin thread after application of ATP without load.

Abscissa: Time in minutes.

Ordinate: Length in per cent (equilibrium length = 100).

has a considerably lower gradient than before application of this substance.

In order to provide information concerning the minute structural changes which occur in the "contractile" myosin thread after application of ATP, we have in a series of experiments determined dynamic stiffness, length and tension before and during the application of ATP. The time course of the changes in length produced by ATP at different loads is given in Fig. 5 for load 0 (tensionless) up to loads which in the untreated fibre cause an elongation of 1 per cent. While the fibre without load shows a shortening of approximately 30 per cent after application of ATP, we find a distinct elongation even with a load which in the untreated fibre only produces an elongation of less than 0.1 per cent. The observed changes in the mechanical properties were rather unexpected in view of the great

shortening capacity of the threads investigated.

Dynamic stiffness (corrected to constant tension) decreases immediately after immersion of the thread in an 2×10^{-6} mol/ml ATP solution on an average by 30 per cent and at the same time its length increases by approximately 20 per cent (Fig. 6). The decrease in stiffness of 30 per cent can only cause an increase in

length of 4 per cent,¹ and therefore, the changes in stiffness cannot account for the elongation observed. With the fibre continuously immersed in ATP, stiffness increases again, passes the initial value after a period of 4—5 minutes and, since there is a slight increase in stiffness before application of ATP, it approaches presumably

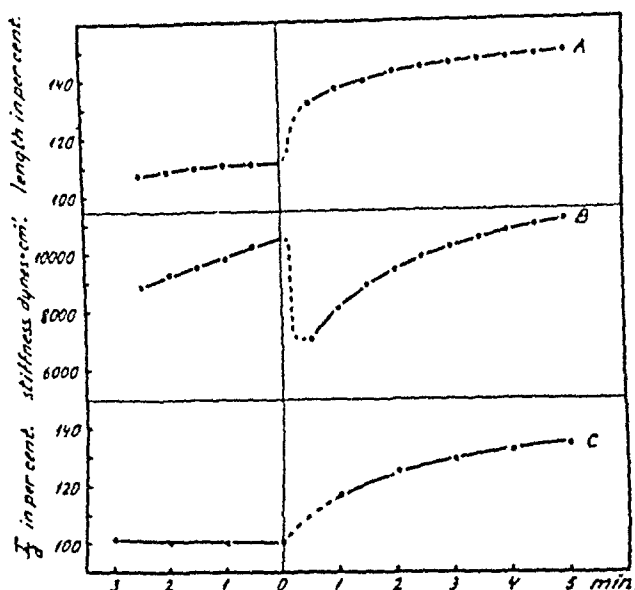


Fig. 6. Changes in length, dynamic stiffness and birefringence caused by ATP (2×10^{-6} mol/ml) in dried myosin threads.

Curve A: Change in length. Ordinate: Length in per cent (equilibrium length = 100).

Curve B: Change in stiffness. Ordinate: stiffness in dynes \times cm⁻¹.

Curve C: Change in birefringence. Ordinate: $\frac{F}{d}$ in per cent.

Abscissa: Time in minutes.

the values which it would have attained without application of this substance. In contradistinction to ENGELHARDT et al. (1941) the alterations produced by ATP in our experiments are independent of the presence of an enzymatic activity.

When stiffness is determined by measuring the changes in length, produced by small periodically varying changes in load, the viscous component of stiffness will cause a phase difference

¹ For a given load the elongation above equilibrium length is inversely proportional to the stiffness. Before application of ATP stiffness is 10,000 dynes \times cm⁻¹ (Fig. 6 B) and the elongation above equilibrium length 10 per cent. Under the influence of ATP stiffness decreases to 7,000 dynes \times cm⁻¹. This produces an elongation of 10 per cent, $\frac{10,000}{7,000} = 1.428$, i. e. the elongation due to the increase of stiffness amounts to 14 per cent—10 per cent = 4 per cent.

in the propagation of the changes in length and tension. In the myosin thread the phase difference at a frequency of 6 cycles per second is much lower than in muscle, which implies that the viscous component of stiffness in the former amounts only to one

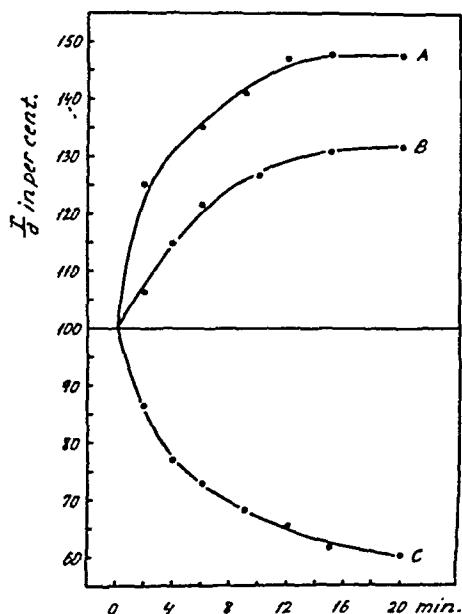


Fig. 7. Changes in birefringence of dried myosin threads after application of ATP (2×10^{-6} mol/ml).

Curve A: Isotonic conditions.
 Curve B: Isometric conditions.
 Curve C: Tensionless.
 Abscissa: Time in minutes.
 Ordinate: $\frac{I}{d}$ in per cent.

third of that found in muscle, referred to the same stiffness.

When the thread is kept under *isotonic conditions*, birefringence increases after application of ATP in the course of 5 minutes approximately 30 per cent and reaches its maximal increase of 40–50 per cent after 15 minutes (Fig. 7 A). In the threads with *load zero*, birefringence decreases simultaneously with the shortening (Fig. 7 C), while it increases in a thread which is kept *isometrically* (Fig. 7 B). This increase is, however, less pronounced than that observed in threads with constant load. If a relaxed thread, both ends of which are fixed, is treated with ATP, birefringence at first decreases simultaneously with a shortening, for a period of approximately 4 minutes. This

reduction is followed by an increase corresponding to the isometric conditions which arise when the fibre shortens and is stretched so much that the desorientation cannot continue (Fig. 8).

Discussion.

The independence of enzymatic activity and physical changes induced by ATP in myosin threads is a further indication of the non-identity of the contractile protein with adenosine triphosphatase (PRICE and CORI (1946), POLIS and MEYERHOF (1946)). Thus, it stresses the assumption, that the primary effect of ATP

on the contractile protein as observed in muscle and myosin is not identical with the enzymatic reaction. Intact sulphhydryl groups previously shown to be necessary for the activity of the enzyme protein (SINGER and BARRON (1944), ZIFF (1944)) are found to be a prerequisite for the release of physical changes by ATP in the contractile protein. In view of these results we have investigated the action of sulphhydryl reagents on the effect of

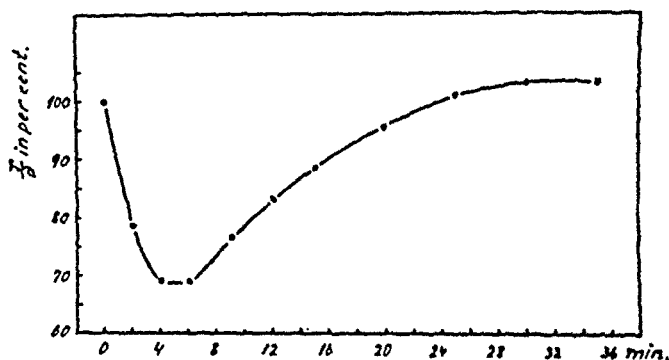


Fig. 8. Change in birefringence in a dried myosin thread after application of ATP (2×10^{-3} mol/ml). The thread is relaxed and both ends are fixed.

Abscissa: Time in minutes.

Ordinate: $\frac{I}{d}$ in per cent.

ATP in the muscle fibre. There too, in a molar concentration of 0.0005–0.001, they inhibit changes in birefringence.

While ATP can be replaced by inosine triphosphate both in the release of contraction in the muscle fibre and as substrate for adenosine triphosphatase in myosin solutions, ATP is the only substance hitherto found which releases the strong volume constriction in the myosin thread. In its action on skeletal muscle adenosine diphosphate is identical with ATP in every respect, just as adenosine + pyrophosphate can replace ATP. None of these have an effect on the myosin thread. In this connection it is of interest to recall the similarity which exists between the reaction of myosin threads and that of smooth muscle, the latter likewise being insensitive to adenosine diphosphate and adenosine + pyrophosphate. Triphosphates other than ATP, viz. inosine triphosphate and inorganic triphosphate have been shown to release contraction in skeletal muscle. In myosin threads the initiation of the physical changes demands the presence of both the triphosphate and the intact purine riboside part of ATP. The reaction of the

triphosphate group with the protein manifests itself in an inhibitory action reminding of the competitive inhibition which triphosphate has on the adenosine triphosphatase in myosin solutions (DAINTY et al. 1944).

It is uncertain, whether the protein constituting myosin threads is identical with the contractile protein in muscle. Apart from the differences in the effect of ATP and related compounds on myosin threads and on the muscle fibre, the structural changes, which occur during the volume constriction of the myosin thread, are fundamentally different from those observed in the muscle fibre.

The reverse changes in length produced by ATP in threads with small loads and load zero can only be explained assuming simultaneous changes in the elasticity modulus and the equilibrium length of the substance. This assumption is confirmed by direct measurements of stiffness which in contrast to findings in muscle decreases simultaneously with an increase in length. The elongation being considerably larger than that which would arise from the decrease in stiffness observed, also indicates a change in equilibrium length. Determination of dynamic elasticity in the myosin thread gives no indication of the presence of a viscous component in the resulting stiffness at a frequency of 6 cycles per second. In muscle it is found and is assumed to be due to linkage modifications in the contractile elements.

The increase in birefringence after application of ATP in a thread which is kept under isometric conditions indicates that birefringence increases as soon as the thread is exposed to tension, even if the latter is slowly reduced. The increase which presumably is due both to a change in crystalline and form birefringence is masked in the thread at load zero by a decrease in birefringence accompanying the strong desorientation.

In terms of minute structure the mechanical changes which occur in a myosin thread after application of ATP must involve the release of a certain proportion of elastically active molecular or micellar linkages, *i. e.* probably a partial dissolution of certain structural elements. The elimination of approximately two thirds of the elastically active elements would account for the mechanical changes observed. The changes in the mechanical properties of a myosin thread after application of ATP are reverse to those occurring in muscle during contraction and indicate that the volume constriction in a myosin thread from the point of view of minute

structural changes cannot be considered a model of the process of contraction.

Summary.

In confirmation of SZENT-GYÖRGYI et al. we find that ATP causes a strong volume constriction in myosin threads. This effect occurs in threads prepared both from enzymatically active and practically inactive myosin.

Adenosine diphosphate and inosine triphosphate which release contraction in the muscle fibre, are without effect on the myosin thread.

Birefringence decreases considerably during the shortening produced by ATP, while it increases when shortening is prevented.

Sulphydryl reagents reduce the changes in volume and birefringence produced by ATP in the myosin thread and also make a muscle fibre less excitable by ATP and electrical stimuli.

Creatine phosphate, sodium triphosphate, sodium pyrophosphate and sodium ortophosphate, ineffective as such, likewise reduce the effect of ATP, while it is enhanced by inosine triphosphate.

Previous treatment with acetylcholine reduces the effect of ATP only in enzymatically inactive dried myosin threads.

Threads which show a strong shortening ability towards ATP in a tensionless condition, react with a considerable decrease in stiffness and increase in equilibrium length even at loads which in the untreated thread only produce a stretch of 0.1 per cent. Both effects are interpreted as being due to the release of a certain proportion of elastically active linkages in the minute structural elements by ATP.

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Investigations Concerning the Utilization of Ketone Bodies during Muscular Exercise.

By

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Introduction.

Man is able to perform vigorous muscular exercise under circumstances, where the chief source of energy must be fats, *e. g.* when living on a diet without carbohydrates, or especially rich in fats and poor in carbohydrates, or after starvation for some time when the carbohydrate stores of the organism have run low. It has therefore been discussed for a long time how the energy requirement of the muscular tissue is met under these circumstances.

After the researches of LEATHES (1906) and WIENFIELD (1915) it was assumed that the muscles could not utilize the fatty acids directly, and the ability of fats to provide energy for exercise was explained by a conversion of fatty acids into either carbohydrate or ketone bodies before the combustion in the muscles.

In 1938 BLIXENKRONE-MØLLER demonstrated that ketone bodies are able to provide energy for the contraction of muscles and LUNDSGAARD (1941) therefore claims that striated muscles may use ketone bodies at rest as well as during work.

It has been the object of the present investigation to find out whether the blood of man during exercise contains ketone bodies in such quantities as to make it probable that all the fat which according to the respiratory quotient is oxidized in the muscles, could have been offered in the form of ketone bodies.

This question has not previously been the subject of experimental investigation.

The literature contains several statements as to the quantity of ketone bodies in the blood of man and its variation during exercise and after. The main result of previous investigations is that the concentration of ketone bodies in the blood of man is unchanged or somewhat reduced at the end of work and increased during the first few hours after its cessation. However, it is regrettable that the previous investigations (except those of ASMUSSEN, WILSON and DILL (1940)) have been confined to the determination of ketone bodies in the blood after the cessation of exercise. It appears evident that the blood must be examined *during* exercise in order to yield information regarding the changing concentrations of ketone bodies due to muscular exercise and it is necessary to determine the dispersion of results in order to judge the fluctuations which may be observed. The analytic errors in relation to the variation in the ketone bodies have not previously been made the subject of discussion except in one single case (HIMWICH, LOEBEL and BARR (1924)).

Subjects and procedure.

The subjects were two men, O. H. weight about 70 kgs. and age about 34 years, and B. H. weight about 65 kgs. and age about 28 years. Both were trained to the work on a KROGH bicycle ergometer and to respiration through a mouthpiece and valves.

The subjects came to the laboratory in the morning before taking any food and rested for 8 minutes without the mouthpiece and then for 15 minutes breathing through a mouthpiece and valves. The resting metabolism was determined by means of DOUGLAS bags. Immediately afterwards the first blood sample was drawn to estimate the ketone bodies and as a rule also the blood sugar. The technique used in drawing the blood samples is similar to that described by HOHWÜ CHRISTENSEN, KROGH and LINDHARD (1935). All the blood samples were collected from the finger tips, the hand having been definitely hyperemized in water of about 45° C. The prick was made with an "automatic" lancet forcing its way a couple of mm through the skin. The blood either flowed spontaneously or else it was pressed out by a milking movement with the other hand. The first drop was wiped off and then one slightly concave end of a small celluloid tube (covered with a thin layer of sticking plaster substance so as to make it adhesive) was quickly applied to the bleeding spot. The blood rapidly rose into the tube by means of continued "milking" and when a suitable quantity of blood had been collected into the tube the blood sample was removed with a pipette

(0.5 cc). The subject thereafter performed some arm work which is of no interest in connection with the present paper. Directly after that the second blood sample was collected and thereupon (as a rule 5—8 min. after the cessation of the arm work) the leg work was started on a KROGH bicycle ergometer. B. H. always worked with a load of 720 kilogrammetres per min., O. H. doing 1,080 kgm. per min., both at a rate of 60 revolutions per min. As long as the diet was normal, the exercise was sub-maximal (exp. Nos. I—III). 13 and 43 min. after the start samples were again taken for determination of the metabolism (duplicate analysis), the subject having been breathing through a mouthpiece and valves for at least 2 min. Blood samples (single samples) were also taken in this connection.

After the work period the subject again rested for about 15 min. before the last blood samples were drawn. Urine was voided before and after the experiment.

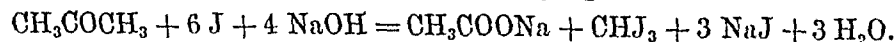
The subject then went about his daily work and returned, still fasting, to the laboratory in the afternoon, when the experiment was repeated. After the second experiment as a rule he had a ketogenic meal composed of cabbage, butter, bacon and apples, and cream, but without carbohydrate, and was fasted until the following day when two identical experiments were carried out. As a rule these last experiments were a great strain on the subject who at the end suffered from nausea, giddiness, headache, dimness of vision and depression.

The air analyses and the calculations of the R. Q. were made by OVE HANSEN, Ph. D., the estimation of the blood sugar by Miss SÛRPLI, and the analyses of the ketone bodies by the author himself.

Method

used for determining the ketone bodies in the blood.

Principle of the method. POULSEN's method (1941) was used in order to estimate the ketone content of the blood. The principle of this method is to oxidize acetoacetic acid and β -hydroxybutyric acid into acetone which together with the free acetone already existing in the blood is collected in an alkaline iodine solution in which the acetone forms iodoform according to the following equation:



The loss of iodine in this solution is determined by titrating the remaining iodine with sodium thiosulphate. The original concentration of the iodine solution being known, and the conversion of a molecule of iodine into iodides requiring two molecules of sodium thiosulphate, the amount of acetone can be calculated (0.4835 gamma of acetone corresponds to 1/100 cc N/200 sodium thiosulphate).

Procedure. Certain modifications having been made in the construction of the distilling apparatus and the process of distillation, the practical performance of the method will be briefly described.

0.5 cc of blood is used for the analysis and at once mixed with 7 cc

of distilled water. 1 cc is used of each of the 3 precipitants: basic lead acetate solution (specific gravity about 1.245), zinc sulphate solution (5 per cent) and sodium hydroxide solution (0.8 N).

The mixture is centrifuged for 20 minutes and 7.5 cc of the centrifuged liquid (*i. e.* 71.4 per cent of the starting material) is transferred to the funnel of the distilling apparatus. The funnel and the glass tap are connected with the distilling flask (volume 35 cc) by a small tube opening into the side wall. In this manner the solutions added through the funnel are prevented from running into the long tube leading from the top of the distilling flask to the receiver such as has happened when working with an apparatus like the POULSEN model with both tubes in the top of the flask.

The receiver contains 5 cc about N/200 iodine solution plus 5 cc 0.75 N sodium hydroxide solution.

2.5 cc of 20 per cent sulphuric acid is poured into the flask before the distillation. The flask is placed in an oil bath heated by gas. By means of a thermostat the temperature is kept between 137° and 138° C with a maximum variation of 1°.

The boiling with sulphuric acid alone is continued for 20 minutes after which period nearly all the acetone originating from free acetone and aceto-acetic acid has been distilled into the receiver (*first fraction*).

The apparatus is now removed from the oil bath, the receiver changed and 5 cc distilled water added to the flask which is again placed in the bath. When the contents of the flask are boiling again a potassium bichromate solution (2.5 cc 0.75 per cent) is added in the manner described by POULSEN. The β -hydroxybutyric acid is oxidized by the chromium-sulphuric acid and partly converted into acetone which is distilled into the receiver in the course of the next 35 minutes (*second fraction*).

After the distillation 2—3 drops of a 1 per cent starch solution and 5 cc 1.5 N sulphuric acid are added to the receiver whereupon titration is immediately performed with N/200 sodium thiosulphate by a 2 cc burette. The result is measured with an accuracy of 1/1000 cc.

Distillation exceeding the periods stated — 20 and 35 minutes respectively for the two fractions — only gives insignificantly increased quantities.

Yield.

The recovery of the different ketone bodies is not quantitative. It averaged 95 per cent with an error of $\pm 3\%$ for acetone and acetoacetic acid. The yield of β -hydroxybutyric acid averaged 68 per cent with an error of ± 4 , *i. e.* about ± 6 per cent of the value.

These dispersions have been found by investigating the systematic errors of the method after introducing 36.3 gammas of β -hydroxybutyric acid into $\frac{1}{2}$ cc of blood (corresponding to 7.3 mg per cent in the blood), $\frac{1}{2}$ cc of blood without such addition being used as a blank. In cases of larger quantities of β -hydroxybutyric acid in the blood the percentage error must be smaller, but it is of no importance to the

calculations given below that a dispersion to 6 per cent is assumed also in cases of larger quantities.

Calculation of total ketone bodies as β -hydroxybutyric acid.

The number of hundredth parts of cc N/200 sodium thiosulphate is converted in acetone by multiplying with 0.4835 (cf. p. 3) and the acetone is converted into β -hydroxybutyric acid by multiplying with $\frac{104}{58}$.

As only 71.4 per cent of the blood sample is used for the analysis and the percentage yields of acetoacetic acid and β -hydroxybutyric acid are 95 and 68 per cent respectively the actual amount of ketone bodies of the blood are arrived at when multiplying the first and second fractions with $\frac{105}{71.4}$ and $\frac{147}{71.4}$ respectively. For practical reasons and out

of regard to the calculations which will be mentioned later the total amount of ketone bodies will be referred to as β -hydroxybutyric acid per litre. Since the molecular weight of acetoacetic acid and β -hydroxybutyric acid is 102 and 104 respectively, the amount of total ketone bodies referred to as β -hydroxybutyric acid is very close to the actual quantity met with in the blood.

Urine.

The urine was examined qualitatively for acetone and acetoacetic acid using LEGAL's and GERHARDT's tests respectively.

Results of the experiments.

The material comprises 12 experiments carried out in January 1942. The results of exp. nos. I, VI and XII are given in the table. The experiments have been arranged according to increasing ketonemia without paying regard to the chronological order.

The table includes statements of the experimental periods (1), their duration in hours and minutes, the beginning of the experiment being reckoned as zero (2), the moments of taking the blood samples (3), their content of acetone and acetoacetic acid (4), β -hydroxybutyric acid (5), and total ketone bodies (6), the results of the analyses being corrected as mentioned before, and the ketone bodies expressed as mg β -hydroxybutyric acid per litre of blood.

The table shows further the average consumption of oxygen, expressed as l per min. (7), the average R. Q. (8), the estimated

blood minute volume of the subject (9), and the estimated blood minute volume of the muscles (10). The blood minute volume of the subject is calculated according to HOHWÜ CHRISTENSEN (1931) from the oxygen consumption during exercise while the resting blood minute volume is estimated at 5 litres.

As a basis for calculations to be mentioned later it is assumed that 40 per cent of the resting minute volume and about 90 per cent of the minute volume during exercise flow through the muscles. As appears from the table the latter entails an increase in minute volume exclusively in favour of the muscles and even a somewhat reduced circulation in the other organs (vessel contraction *e. g.* in the splanchnic region), conveying an extra quantity of blood to the muscles. We take it that this represents the actual distribution of the blood flow as closely as possible.

The minute volume and the concentration of β -hydroxybutyric acid in the blood thus being taken as known it is possible to calculate the quantity of ketone bodies conveyed to the muscles by the blood per minute (14).

From the oxygen intake and CO_2 elimination the amounts of fat and carbohydrate oxidized per minute (11 and 12) may be arrived at on the basis of investigations by ZUNTZ and SCHUMBURG (1901).

The next figure stated in the table is the quantity of β -hydroxybutyric acid presumably oxidized by the muscles per minute (13). This figure is derived from the quantity of fat broken down, assuming that one molecule of palmitic acid is converted into 4 molecules of β -hydroxybutyric acid (BLIXENKRONE-MØLLER, 1938). The loss of energy by this conversion is 17.9 per cent.

KROGH and LINDHARD (1920) find a loss of energy of 10 per cent in exercise experiments where the subject was on a fat diet. The maximal loss of energy in human subjects exercising on a fat diet is 12 per cent (MARSH and MURLIN, 1928).

According to BLIXENKRONE-MØLLER (1938) the rather considerable difference between the theoretically calculated loss of energy and that found experimentally does not exclude the possibility that the fat metabolized is broken down into ketone bodies in this manner. It may, however, be objected to this view that the error in the loss of energy found experimentally can hardly be as large as assumed by BLIXENKRONE-MØLLER. This seems to indicate that hardly all the fatty acid are converted into ketone bodies before the combustion.

When one molecule of fatty acid corresponds to 4 molecules of β -hydroxybutyric acid, 1 gm of fatty acid corresponds to 1.65 g of β -hydroxybutyric acid, assuming that palmitic acid is the only one present in fat. The error due to this assumption is of no account in calculations as rough as the present.

Since the fats contain only 90 per cent fatty acids and 10 per cent glycerine, the quantity of fat found must be reduced by one tenth before the conversion into ketone bodies by multiplying with the factor 1.65.

It is assumed that the total amount of fatty acid catabolized is oxidized in the muscles during exercise and that only one third is used by the resting muscles.

The uncertainty of the calculations.

The uncertainty connected with these calculations is made up of the analytic error in estimating the ketone bodies of the blood (± 6 per cent), the possible error on the minute volume of the muscles (about 20 per cent, cf. CHRISTENSEN 1931, table 9), and the error arising from the assumption that during exercise the breakdown of fat is effected by the muscles alone, the metabolism of which is, however, quite dominant.

The error in the fat metabolism arising from using the unreduced R. Q. instead of the non-protein R. Q. is of no importance. The correction observed in the preliminary experiments was ± 0.004 in R. Q. and the unavoidable uncertainty in R. Q. is 0.02. This error in R. Q. estimation gives rise to an error in the calculated metabolism of fat of about 0.005 g per litre of oxygen which is insignificant.

Thus the dominant source of error will be the uncertainty of the calculation of the minute volume of the muscles. The total error of all calculations is estimated to be about 26 per cent.

The experimental results.

During rest in these experiments the muscles will always be supplied with sufficient ketone bodies to allow the entire amount of fat catabolized per minute to be supplied in the form of ketone bodies. During work the situation is different.

The following will only be a discussion of the conditions during

leg work in the 12 experiments as no determinations were made *during* the arm work.

With the exception of exp. Nos. II and V where the samples drawn after cessation of the arm work have been lost, and exp. No. XI where the difference between resting and working values is no greater than the analytic error, a fall was observed in the concentration of ketone bodies during work followed by a violent and quick increase immediately upon cessation of work.

It is remarkable that most frequently it is the β -hydroxybutyric acid fraction which is increased after cessation of work.

The variations in the blood sugar were estimated in exp. Nos. V—VIII, X, and XI. Excepting the last-mentioned experiment a fall was observed during leg work.

In exp. Nos. VII—XII there was ketonuria before the work corresponding to concentrations of ketone bodies in the blood of above 190 mg/l during rest.

The concentration of ketone bodies — as well as that of the blood sugar — shows a reduction during leg work indicating a consumption in excess of production. The problem now to be discussed is whether this fact means that ketone bodies are the only derivatives of fatty acids supplied to the muscles. The same question may also be put thus: Is it possible that excluding the glycerine the entire amount of fat catabolized per minute can be conveyed to the muscles in the form of ketone bodies? The following contains the answer according to the experiments. These may be divided into 3 groups, the first group comprising exp. Nos. I—IV, the second exp. Nos. V—VII, and the third exp. Nos. VIII—XII. Representative examples have been given in full in the table (exp. Nos. I, VI and XII).

O. H. was the subject in exp. Nos. I, II, IV, VI, VII and X, B. H. in exp. Nos. III, V, VIII, IX, XI and XII. The two subjects on the whole gave the same results in all three groups of experiments.

Experiments I—IV.

Exp. No. I is taken as an example for this group. While the subject in exp. Nos. I—III had been living on a normal diet during the previous days, exp. No. IV was carried out after he had been subjected to a very scanty ketogenic diet and done some exercise of short duration (exp. No. II) on the previous day. The ketone concentration during rest before exercising was 39, 43, 31 and

Experimental periods	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Duration of experiment														
Time of collection of blood samples														
Acetone and acetoacetic acid expressed as mg. per litre of blood														
β -hydroxybutyric acid per litre of blood														
Total ketone bodies. mg. per litre of blood														
Average intake of oxygen, l/min.														
Average RQ														
Total minute volume l/min.														
Minute volume of the muscles l/min.														
Calculated oxidized quantity of fat (mg. per min.)														
Calculated oxidized quantity of carbohydrate (mg. per min.)														
β -hydroxybutyric acid presumed to be oxidized by the muscles (mg. per min.)														
β -hydroxybutyric acid presumed to be conveyed to the muscles (mg. per min.)														

 Exp.
No. I
subject:
O. H.

 Exp.
No. VI
subject:
O. H.

 Exp.
No. XII.
subject:
B. H.

99 mg β -hydroxybutyric acid per litre blood respectively in the 4 experiments. During leg work 47 and later 40 (exp. I), 44 (exp. II), 41 and later 54 (exp. III) and 72 mg (exp. IV) were found.

In exp. Nos. I, II and IV the amount of ketone bodies supplied was smaller than that which should have been catabolized to correspond to the fat as indicated by the determination of metabolism, in exp. No. I 2 hours and 23 minutes after the start of the work even considerably less, but in exp. No. III there was a small surplus so that a utilization of 80 per cent of the amount carried by the arterial blood would suffice.

Owing to the rough character of the calculations (total error about 26 per cent), it can hardly be ruled out that ketone bodies may be the only form in which fatty acids are supplied to the working muscles in these experiments, but it seems rather improbable since it involves a utilization of 80 to 100 per cent.

Unfortunately it has not been investigated whether the venous blood from a working muscle or group of muscles actually did contain so greatly reduced quantities of ketone bodies in relation to the arterial blood.

Experiments V—VII.

Exp. No. VI is given as an example for this group. The first two experiments were carried out after about twenty-four hours' starvation and about 5 hours after the end of a previous experiment. The increase in the ketone content of the blood following an exertion reaches its maximum in the course of a quarter of an hour and about two hours after cessation of work the concentration curve of ketone bodies of the blood has adopted an almost horizontal course (see diagram). Therefore the resting value 5 hours after the end of a previous experiment is not directly affected. Exp. No. VII was made the day after a day of two experiments and starvation except for a ketogenic supper.

During rest before the exertion the concentrations of the ketone bodies in these 3 experiments were 83, 120 and 268 mg β -hydroxybutyric acid per litre blood. During leg work 94 and later 120 mg (exp. No. V), 109 and later 135 mg (exp. No. VI) and 140 and later 183 mg (exp. No. VII) were found.

In these experiments a utilization of 44 to 67 per cent (averaging 55 per cent) of the amount of ketone bodies carried by the arterial blood is calculated and therefore the possibility exists that the muscles have received all the fats in the form of ketone bodies.

In exp. Nos. V—VII the blood sugar showed the following variations:

	Rest	Leg work
exp. V	81 mg per cent	85 and later 85 mg per cent
exp. VI	100 » » »	68 and later 85 » » »
exp. VII		48 and later 48 » » »

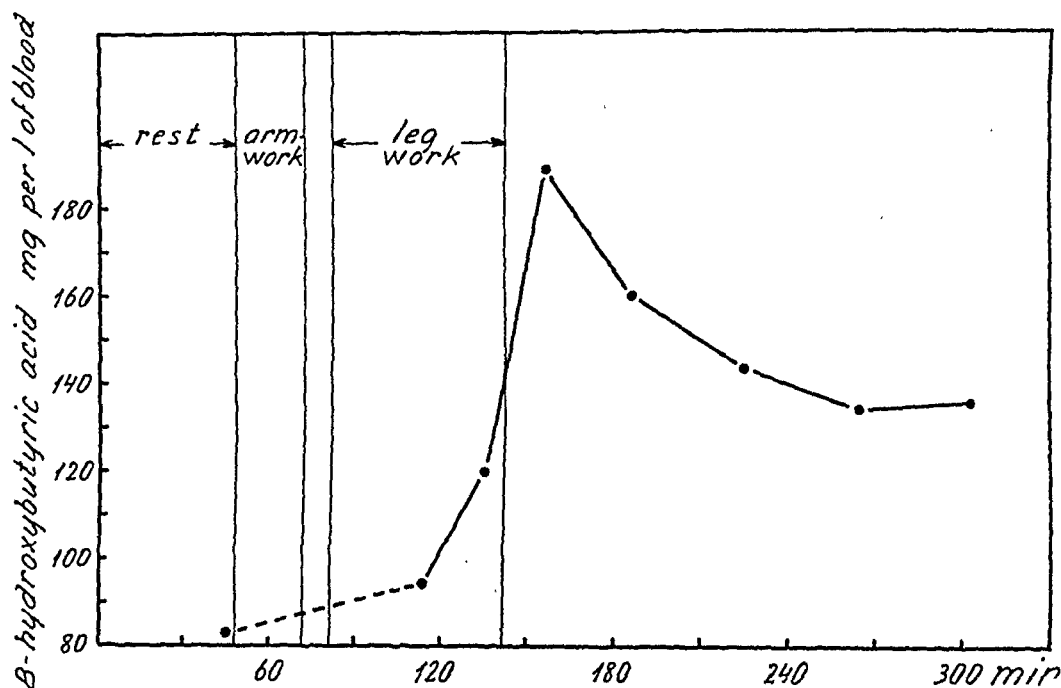


Diagram showing exp. No. V.

Experiments VIII—XII.

Exp. No. XII is taken as an example for this group. All these experiments were carried out under the same conditions as exp. No. VII.

The resting values for the ketone bodies of the blood were 189, 224, 390, 315 and 422 mg β -hydroxybutyric acid per litre blood, the leg work values were 172 and later 223 mg (exp. No. VIII), 176 and later 249 mg (exp. No. IX), 320 and later 314 mg (exp. No. X), 328 and later 326 mg (exp. No. XI), 394 and later 430 mg (exp. No. XII).

During leg work in these experiments the utilization varies between 17 and 41 per cent (averaging 27).

It is possible that the entire amount of fatty acids was supplied to the working muscles in the form of ketone bodies in these 5 experiments.

The concentrations of blood sugar in exp. Nos. VIII, X and XI were:

	Rest		Leg work
exp. VIII	79 mg per cent	79 and later	68 mg per cent
exp. X	68 » » »	54	» » »
exp. XI	63 » » »	67	» » »

Discussion.

The high utilization found in the first 7 experiments makes it difficult to accept the theory that fatty acids must always be oxidized into ketone bodies by the liver before the utilization by the muscles. According to the experiments given above the fatty acids must be utilized by the muscles also in a form other than ketone bodies, perhaps directly without conversion.

In this connection it is interesting to call to mind the above-mentioned, considerable difference between the calculated loss of energy by the conversion of fatty acids into ketone bodies and the actual loss of energy revealed by the experiments. As already mentioned the difference may be explained by assuming that only a fraction of the fatty acids is converted into ketone bodies before the oxidation while another fraction is catabolized without or with a lower loss of energy. This is probably the case, if the last-mentioned fraction of the fatty acids is oxidized directly without preparatory conversion into ketone bodies. This theoretical explanation is supported by the first 7 experiments.

On the other hand, the high concentration of ketone bodies of the blood and the low utilization (about 27 per cent) in the last 5 experiments make it possible to assume that in these the fatty acids have been conveyed to the muscles exclusively in the form of ketone bodies. It cannot, however, be ruled out that also in these cases some fatty acids may have been oxidized directly.

This result of the investigations could fit the view that the liver does not produce ketone bodies in order to supply the working muscles, but that its ketone production becomes an expression for the material on which the liver is dependent in order to obtain the energy for its own metabolism (SOMOGYI, 1941).

In the experiments after a normal diet the liver is rich in glycogen and therefore mainly burns carbohydrate, its preferred source of energy. Its production of ketone bodies is therefore slight and insufficient to cover the fat metabolism of the muscles.

In the later experiments, however, in which the blood sugar is also low, the liver is poor in glycogen and therefore must convert fatty acids into ketone bodies which increase the ketone content of the blood. Hereby the amount of ketone bodies catabolized in the muscles is gradually increased on account of the increasing accumulation in the blood, and at last the muscles receive such an amount of ketone bodies that the latter can become their dominant or only source of energy from fat.

The quick increase of ketone bodies in the blood after cessation of work can mean that during work there has been a large production which cannot be interrupted immediately, or it may be a phenomenon of recovery. The latter may be explained when remembering the very low R. Q. after the exertion observed by previous investigators (BRAND and KROGH, 1935), which low figure may signify a large combustion of fat and a slight oxidation of carbohydrate. May be the increase in the ketone concentration after the exertion is another implication of the efforts of the organism, especially of the liver, to economize as much as possible with carbohydrate after exertion.

Like the investigations by KROGH and LINDHARD (1920) the experiments given above showed a reduced mechanical efficiency in subjects living on a fat diet. The mechanical efficiency found will be discussed by OVE HANSEN, Ph. D., in a later paper. On an average the energy loss was of the same order of magnitude as that found by KROGH and LINDHARD.

Summary and Conclusions.

Previous investigators have found no change or a slight reduction in the ketone content of the blood at the end of work and increased quantities in the first hours after its cessation.

It is attempted to elucidate the question whether the blood of exercising individuals contains ketone bodies in such quantities as to justify the belief that the entire amount of fatty acids oxidized in the muscles has been supplied in the form of ketone bodies.

The concentration of the ketone bodies in the blood is determined in two adult men, exercising on a KROGH bicycle ergometer (load 720 and 1,080 kgm/min.).

The amount of ketone bodies in the blood was estimated by means of POULSEN's method with a few modifications. The procedure and the calculation of total ketone bodies as β -hydroxybutyric acid are described.

The supply of ketone bodies carried to the muscles during work is calculated and compared with the amount of fatty acids catabolized according to the oxygen intake and the R. Q.

Suffering from an increasing ketonemia the subjects went through 12 periods of fairly severe work. When the ketone content of the blood is low (exp. Nos. I—IV), the amount of the ketone bodies found as conveyed is about equal to the quantity calculated as oxidized, i. e. a utilization of about 100 per cent or more must be assumed. It is therefore considered improbable that ketone bodies are the only form in which the fatty acids are conveyed to the muscles in these circumstances. If part of the fatty acids can be metabolized without a previous conversion into ketone bodies and consequently with less loss of energy than demanded by the conversion into ketone bodies (17.9 per cent) it explains the fact that the loss of energy from fats, deduced from careful experiments (9—12 per cent) is lower than this figure.

When the ketone content of the blood is increased, the percentage utilization to be assumed is reduced, so that it becomes possible that ketone bodies can be the only source of fat metabolism in the muscles. However direct determinations of the energy loss in such cases do not seem to confirm this assumption.

The production of ketone bodies in the liver does not therefore seem to be correlated with the fat metabolism of the muscles. The experiments support the view that the production of ketone bodies is dependent on the material at the disposal of the liver. When the fat metabolism in the liver is high, the production of ketone bodies can rise to such heights as to cover the fat metabolism of the muscles entirely.

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Über die Quantitative Wirkung des Pufferner- venmechanismus auf die Blutdruckaus- wirkungen des Adrenalins.

Von

H. KUMPAS.

Eingegangen am 28. November 1946.

Bald nach den Mitteilungen Herings über den Mechanismus der Puffernerven kam STROSS (1928) auf den Gedanken in diesem Mechanismus denjenigen Faktor zu suchen, in dem vielleicht die Ursache zu suchen ist, weshalb die pressorische Wirkung der Zentralanaleptika im Blutdrucktest sich so unsicher darstellen lässt. Seine Untersuchungsergebnisse mit Coffein und Strychnin jedoch fielen in dieser Hinsicht negativ aus. Später haben PALME (1936) und HAHN (1941) die Versuche mit Cardiazol wiederholt. PALME gibt den Puffernerven ihre volle Bedeutung, HAHN jedoch konnte nur einige Male die Potenzierung der manifesten Pressoeffekten des Cardiazols nach Ausschaltung der Puffernerven konstatieren. Dass eine Cardiazoldosis, die bei intaktem Puffermechanismus keine Blutdrucksteigerung ergab, später, nach Ausschaltung des Puffermechanismus eine Blutdruckerhöhung ergeben hätte, konnte HAHN nicht beweisen und hat auch PALME nicht gezeigt.

Über die peripherangreifende kreislaufaktiven Pharmaka liegen keine derartigen Untersuchungen vor. Im Vorliegenden sind die Untersuchungen in dieser Richtung mit Adrenalin vorgenommen.

OLIVER und SCHÄFER (1895) vermerkten, dass die Höhe des Blutdruckausschlages bei ein und derselben Dosis von Adrenalin nach der Vagotomie oder nach Atropin grösser war. Dieses ist später auch von anderen Autoren bestätigt und wie von OLIVER

und SCHÄFER erläutert, ebenfalls durch den Fortfall der vagalen Hemmung des Herzens erklärt. Da, wie jetzt seit HEYMANS (1929, 1930) bekannt, diese vagale Hemmung reflektorisch ist und durch die Puffernerven vermittelt wird, so wäre die Aktion des Puffernervenmechanismus zu den Blutdruckeffekten des Adrenalins in dem Masse auch quantitativ wahrscheinlich, wie sie durch die vagale Hemmung des Herzens bedingt sein könnte.

Was das quantitative zur Geltung kommen der vasodepressorischen Reflexe betrifft, scheinen derartige Beobachtungen nicht veröffentlicht worden zu sein. Doch ist bekannt, dass diese Reflexe während der Wirkungsdauer des Adrenalins vermittelt elektrischer Reizung hervorgerufen werden können, jedoch nur in gewisser Wechselbeziehung zur Dosis. Nämlich fanden OLIVER und SCHÄFER (loc. cit.), CYON (1899), VERWORN (1903), v. D. VELDEN (1906) und OSWALD (1916), dass die Reizung des N. depressor auf elektrischem Wege beim Scheitelpunkt der Wirkung des Adrenalins sowohl in Bezug auf das Vaskulargebiet wie auch des Herzens erfolglos war. SOLLMANN und PILCHER (1912) erwähnen, dass der Depressorreflex durch Adrenalin abgeschwächt wird und nach HUGGETT und MELLANBY (1924) war dieser Reflex bei kleinen Dosierungen vorhanden. Bei den Sinusnerven bestätigte HERING (1925) die gleichen Verhältnisse. Bei kleinen Dosen seien die Reflexe nicht schwächer. Bei etwas grösseren Dosen verringere sich die Reaktion augenscheinlich, bis schliesslich bei grossen Dosen weder der Kardial- noch der Vaskularreflex ausgelöst werden kann.

GERNANDT, LILJESTRAND und ZOTTERMAN (1946) fanden auf der Höhe der Adrenalinwirkung sowie kurz nach der Unterbrechung der Asphyxie, während der Blutdruck gerade zu fallen begann aber noch hoch lag, dass die elektrische Aktivität im N. splanchnicus schwächer als im normalen Zustande oder sogar vollständig verschwunden vorgefunden wurde. Nach dem Durchschneiden der Puffernerven konnte während der Adrenalinwirkung kaum eine Andeutung in Richtung der Abnahme der elektrischen Aktivität im N. splanchnicus festgehalten werden. OLIVER und SCHÄFER (loc. cit.) beschreiben, dass der Effekt der Reizung des zentralen Stumpfes des N. depressor während einer starken Blutdruckwirkung des Suprarenalextraktes allmählich wieder in Erscheinung trat, demgemäss wie der Blutdruck sich dem normalen Niveau näherte. Hierzu gehört auch der Befund von HERING (loc. cit.), dass im Beginne eines starken Blutdruckan-

stieges noch eine reflektorische Senkung infolge elektrischer Reizung eines Puffernerves möglich war. Wenn aber der Blutdruck im Fallen war, trat kein Erfolg mehr ein, obwohl die Reizung von derselben Höhe des Blutdruckes am auf- und absteigenden Schenkel des Blutdruckeffektes vorgenommen wurde. GERNANDT, LILJESTRAND und ZOTTERMAN betrachten die im N. splanchnicus beobachtete elektrische Inaktivität nebst Abnahme der chemischen Reizungen seitens der sino-aortalen Rezeptoren bei Adrenalin, hauptsächlich als Folge der von den Pressorezeptoren ausgeübten sehr starken Hemmung auf das vasomotorische Zentrum, und im geringeren Masse als Folge der verringerten Reizbarkeit des Zentrums, welche auf eine sehr intensive Aktivität folgt. Zu den Befunden von GERNANDT, LILJESTRAND und ZOTTERMAN dürften sich diejenigen von OLIVER und SCHÄFER und HERING zur gemeinsamen Fassung in dem Sinne anschliessen, dass im Falle dieser Autoren das Zentrum unter der Einwirkung der peripheren Reflexe stand, da nicht alle Puffernerven durchgeschnitten waren.

Wenn nun die autonomen Kreislaufreflexe, die, ausgelöst durch die Blutdrucksteigerung des Adrenalins, ihrerseits und gleichzeitig dieser Blutdrucksteigerung entgegen ständen und sie zurück hielten und solcherart quantitativ zur Geltung kämen, so müsste beim Vergleich der Blutdruckausschläge vor und nach der Ausschaltung des Puffermechanismus die stattgefundene Gegenregulation als Differenz in Erscheinung treten. Um so viel, wie der Blutdruckausschlag der gegebenen Dosis sich nach Eliminierung der Pufferreflexe vergrössern würde, wäre das Mass der stattgefundenen effektiven Gegenregulation gegeben.

Aus der Blutdruck-Herzfrequenz-Charakteristik von KOCH (1931), LIM und HSU (1931), SCHNEYER (1934, 1935) und WINDER (1938) ist ersichtlich, dass der Reflexerfolg bei zunehmendem Druck zunimmt. Bei Einwirkung von Adrenalin sollte sich demgemäss der Reflexerfolg der Höhe der Blutdrucksteigerung bzw. der Grösse der Dosis entsprechend vergrössern. Andererseits fände unter Berücksichtigung der Beobachtungen bei der elektrischen Reizung und dem Verhalten der elektrischen Aktivität im N. splanchnicus während der Adrenalinwirkung eine Abschwächung der kardio-vaskularen Reflexe ebenfalls in Richtung der Vergrösserung der Dosis statt. Die Stärke der Reizung der Puffernerven (die im gegebenen Falle von der Höhe des Adrenalinblutdruckeffektes abhängig ist) und die gleichzeitige Schwächung des reflektorischen Erfolges (die von der Grösse der Dosis abhängig wäre) wür-

den also die beiden Faktoren sein, von deren dynamischer Stellung zu einander bei der gegebenen Dosis das zur Wirkung kommende voraussichtliche Mass der kardio-vaskularen Gegenregulation, gemessen als Differenz der Höhe der Blutdruckeffekte, vor und nach Eliminierung des Mechanismus der Gegenregulation, abhängig wäre.

Vorbemerkungen zur Versuchstechnik.

Aus der obengebrachten Analyse ist ersichtlich, dass, wenn die Pufferreflexe in den Blutdruckeffekten des Adrenalins quantitativ zur Wirkung gelangen sollten, dann bei verschiedenen Dosierungsbereichen in verschiedenem Masse. Da die von der Dosisgrösse abhängende Abschwächung des Reflexes bei einer gegebenen Dosis von Tier zu Tier verschieden sein kann, sind die Versuche mit einer einzigen Dosis nicht zulässig um über das quantitative Eingreifen der autonomen Reflexe im allgemeinen zu urteilen, sondern an Hand einer steigenden Dosierungsserie ist das Problem zu erleuchten. Im Vorliegenden sind die Resultate individuell an Einzeltieren in Erfahrung gebracht und analysiert worden. Die Methoden einer summarischen Statistik wurden wegen der gegenseitigen Verdeckung der Details beiseite gelassen.

Eine Voraussetzung der quantitativen Untersuchungen über die Wirkung des Puffermechanismus auf die Höhe des Blutdruckeffektes des Adrenalins ist das Konstantbleiben sämtlicher übrigen Faktoren, von denen die Höhe des Blutdruckeffektes des Adrenalins abhängig sein könnte. Wie aus der Literatur ersichtlich, ist diese von der molaren Konzentration der zu injizierenden Lösungen, der Applikationsstelle und der Schnelligkeit der Injektion abhängig. Sie hängt von der Ventilation, des pH des Blutes und der Tiefe der Narkose ab und ändert sich mit Änderungen der Blutmenge. Wenn wir annehmen, dass alle diese Faktoren in befriedigendem Masse kontrollierbar sind, dann müssten, entsprechend dem Zweck der vorliegenden Arbeit, noch die nachfolgenden Umstände betont werden. Vor allem die Höhe des Ausgangsblutdruckes, von dem die untereinander zu vergleichenden Blutdruckeffekte ausgehen. Bei höherem Ausgangsblutdruck kann ein und dieselbe Dosis Adrenalin einen niedrigeren Blutdruckausschlag geben, als bei niedrigerem Ausgangsblutdruck. Aus diesem Grunde sind nur diese von einem und demselben Ausgangsblutdruck ausgehenden Blutdruckausschläge des Adrenalins vor und nach Ausschaltung des Puffernervenmechanismus quantitativ untereinander abschätzbar. Wenn man aber den Erscheinungsverlauf an der Serie der steigenden Dosen verfolgen und auswerten will, wie es im Vorliegenden geschehen ist, dann muss als *conditio sine qua non* angesehen werden, dass die Blutdruckeffekte sämtlicher Dosierungen von einem und demselben Ausgangsblutdruck ausgehen. Hierbei aber treten störende Umstände auf: beim Kaninchen beginnt schon von kleineren Dosen (1—2 γ /kg) an dem Erlöschen des Pressoeffektes des Adrenalins die Senkungsphase des

Blutdruckes zu folgen. Diese Nachsenkung nimmt zu bei Vergrößerung der Dosis sowohl in Bezug auf die Tiefe wie auch auf die Dauer. Bei den vorliegenden Versuchen wurde jede folgende Dosis verabreicht, nachdem der Blutdruck von der genannten Nachsenkung wieder auf das Ausgangsniveau gestiegen war. Die Fälle, bei denen der Blutdruck sich von der Nachsenkung nicht erholte, oder aber mit jeder folgenden Dosis von Mal zu Mal mehr deprimiert wurde, sind bei der Beurteilung der Versuchsserien unberücksichtigt gelassen.

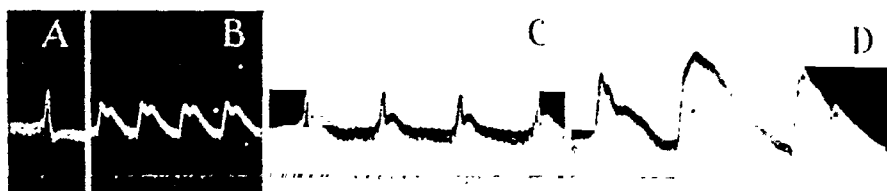


Abb. 1. Kaninchen, Gewicht 1.75 kg, vagotomiert, Sinusnerven und Depressoren intakt. Zeitschreibung 10 Sek.

- A. Blutdruckeffekt nach erster Einspritzung von 0.6 γ /kg Adrenalin.
- B. Injektionen nach je 30 Sek. ergeben niedrigere Blutdruckausschläge.
- C. Die gleiche Dosis nach 1-minütigen Zwischenpausen injiziert, ergab Blutdruckeffekte gleicher Höhe wie bei A.
- D. Vergrößerung mit nachfolgender Abnahme des Blutdruckeffektes bei 3 γ /kg Adrenalin. Die Injektionen erfolgten unmittelbar nach jedesmaligem Absinken des Blutdruckes auf das Ausgangsniveau.

Diese Nachsenkung verschwinde nach v. EULER (1938) mit der Vagotomie aber nicht mit Durchschneidung der Puffernerven. Der Autor der vorliegenden Arbeit konnte das Gegenteil konstatieren: diese Nachsenkung verschwand nicht nach der Vagotomie jedoch aber nach Durchschneiden sämtlicher Puffernerven. Würde auch die Nachsenkung nach der Vagotomie manchmal weniger hervorragend zu Tage zu treten scheinen, kann diese in Beziehung der obendiskutierten Auswertung der Blutdruckausschläge nicht als erleichternder Umstand zur Ausnutzung kommen. Abb. 1 zeigt, dass, wenn die gleiche Dosis von Adrenalin nach kurzen Zwischenpausen wiederholt wird, sein Blutdruckeffekt vergrößert oder verkleinert zu Tage treten kann. Bei kleinen Dosierungen konnte eine Verminderung beobachtet werden (s. Abb. 1-B), jedoch bei grösseren wurde bei der ersten Wiederholung stets eine Erhöhung des Blutdruckeffektes erhalten, der wiederum eine Abnahme folgen konnte (s. Abb. 1-D). Wenn die gleiche Dosis nach längeren Pausen injiziert wurde, wie aus Abb. 1-C bei 0.6 γ /kg ersichtlich, hatten die Blutdruckeffekte ihre normale Höhe. Bei grösseren Dosen muss diese Pause länger sein. Diese beschriebenen Veränderungen in der Höhe des Blutdruckeffektes des Adrenalins in Verbindung mit der Wiederholung der Dosis, treten nicht mehr in Erscheinung, wenn die Puffernerven durchschnitten sind. Es sei hinzugefügt, dass es dem Autor nicht bekannt ist, dass die letztgenannten Verhältnisse früher beschrieben seien.

Methode.

Als Versuchstiere wurden Kaninchen mit einem Körpergewicht von 1.7—3.8 kg benutzt, die mit 1.4 g Urethan auf je 1 kg des Körpergewichtes narkotisiert waren, das in die Ohren-Marginalvene injiziert wurde. Die Versuchstiere atmeten spontan durch die Trachealkanüle die Luft des Aufenthaltsraumes. Das Adrenalin wurde zwischen einer in die V. femoralis eingeführten Kanüle und eine mit Gradzeichen versehenen Bürette in einen Gummischlauch injiziert, von wo das Adrenalin in 4 ml Ringerschen Lösung in den Blutkreislauf eingespült wurde. Die Infusionsgeschwindigkeit war kontrolliert und dadurch gleichmässig gehalten, dass die Bürette nach jeder Infusion wieder bis auf den vorigen Stand nachgefüllt wurde. Die Infusionsgeschwindigkeit schwankte von Tier zu Tier zwischen 4—5 Sek., was durch Zeitsignale geprüft wurde. Die Injektionen wurden mit einer, bis auf 0.01 ml genauen Spritze vorgenommen. Um den möglichen, von der Ungenauigkeit der Injektionsspritze herrührenden Fehler auszugleichen, wurden folgende Verdünnungen angewandt: 1 : 4 000 000 bei Dosen von 0.025 und 0.05 γ /kg, 1 : 400 000 bei 0.1, 0.3 und 0.6 γ /kg, 1 : 40 000 bei Dosen bis zu 6 γ /kg incl. und eine Verdünnung von 1 : 10 000 bei allen grösseren Dosen. Die Verdünnungen wurden mit destilliertem Wasser gemacht. Der Blutdruck wurde von der A. femoralis auf berusstes Papier registriert. Zur Verhinderung der Gerinnung wurde Heparin in die Arterienkanüle eingespritzt. Die Blutdruckausschläge wurden vom Ausgangsblutdruck aus gemessen und nur vom gleichen Ausgangsblutdruck erhaltene Blutdruckausschläge wurden, bzw. vor und nach Ausschalten des Puffermechanismus, für gegenseitig vergleichbar angesehen. Die Sinuse wurden auf dem Wege entnervt, dass das interkarotidiale Gewebe (einschliesslich der A. carotis int.) zwischen doppelte Ligaturen genommen und durchschnitten wurde. Die isolierte Durchschneidung des N. depressor und Vagus wurde im Vagolaryngealwinkel vorgenommen.

Resultate.

I. Die Puffernerven und die minimale Wirkungs-dosis.

Vor dem Versuch wurde jedes Tier vagotomiert und der Versuch begann 20 Min. nach der Vagotomie. Die minimale Wirkungs-dosis wurde auf folgende Weise festgestellt: Die in Tab. 1 angegebene Höhe der Blutdruckeffekte der Injektionsreihe der steigenden Dosierungen des Adrenalins wurden mit der auf die Infusion von 4 ml Ringerschen Lösung folgenden Blutdrucksteigerung verglichen, da die gegebenen Mengen Adrenalin zugleich mit Ringerschen Lösung von dieser Menge in das Blut eingeführt wurden. Als minimale Wirkungs-dosis wurde, entsprechend vor und nach

Tabelle 1.

Verhalten der minimalen Wirkungs-dosis des Adrenalins in Beziehung zum Puffernervenmechanismus.

Versuche an vagotomierten Tieren.

Datum des Versuches/Gewicht des Kaninchens in kg.	Puffernerven erhalten.						Puffernerven durchgeschnitten.							
	Ausgangsblutdr. in mm Hg.	Höhe des Blutdruckauschlages in mm Hg.					Ausgangsblutdr. in mm Hg.	Höhe des Blutdruckauschlages in mm Hg.						
		Ringer 4ml	Dosis in γ /kg.					Ringer 4ml	Dosis in γ /kg.					
			0.025	0.05	0.1	0.3	0.6		0.025	0.05	0.1	0.3	0.6	
14. 6. 45. / 2.1	96	3	3	3	4	6	9	98	3	3	3	3	6	10
7. 9. 45. / 1.8	75	4	—	—	11	14	20	77	6	6	5	9	14	19
16. 7. 45. / 2.6	75	8	—	—	10	15	15	80	7	—	—	8	12	12
25. 6. 45. / 2.9	102	0	0	0	0	6	9	103	4	4	3	6	8	14
11. 6. 45. / 2.6	92	10	10	9	10	10	11	92	18	18	18	21	25	30
29. 8. 45. / 2.4	104	4	3	3	4	4	4	98	14	14	14	18	24	30
27. 8. 45. / 3.7	82	4	4	4	4	5	14	90	14	14	18	22	28	36
17. 8. 45. / 2.0	100	5	4	5	5	9	15	102	10	11	11	24	36	39
22. 8. 45. / 1.9	94	6	7	8	6	8	14	90	16	17	17	20	24	29
11. 7. 45. / 2.4	88	8	6	7	6	6	9	90	3	5	4	11	14	17
5. 7. 45. / 2.5	77	3	3	3	3	9	17	79	2	0	2	8	10	18
23. 7. 46. / 1.7	82	5	3	5	8	18	22	80	4	5	10	19	25	30
31. 1. 45. / 3.2	90	6	6	6	5	8	10	92	6	6	7	10	14	20
19. 9. 45. / 2.0	86	3	3	3	3	6	9	86	8	8	7	11	12	15
21. 9. 45. / 2.3	96	4	4	3	4	4	4	96	13	12	12	12	15	20
25. 7. 45. / 2.0	76	5	4	5	6	17	25	80	10	11	10	15	24	30

Beseitigung der Puffernerven, diejenige kleinste Dosis gerechnet, deren Blutdruckausschlag grösser als der der Ringerschen Lösung war. In der Tab. 1 sind die Blutdruckeffekte kursivgedruckt, die sich als minimale Wirkungs-dosen ergaben.

Die Ergebnisse waren bei den einzelnen Tieren verschieden und sie werden in zwei Gruppen betrachtet, die in der Tabelle durch einen Zwischenraum getrennt sind. Bei Fällen, die in der Tabelle oberhalb dieser Linie angeführt sind, ergab sich als minimale Wirkungs-dosis nach Entfernung der Puffernerven die gleiche Dosis, wie sie bei intakten Puffernerven gewesen war. Die minimale Wirkungs-dosis war nicht maskiert. Nach Beseitigung der Puffernerven konnte keine Vergrößerung der Blutdruckeffekte beobachtet werden. Bei den anderen Fällen, die in der Mehrzahl waren und unterhalb der genannten Wagerechte angegeben sind, war die minimale Wirkungs-dosis maskiert. Die Verschiebung der minimalen Wirkungs-dosis nach Durchschneidung der Puffernerven zur kleineren

Dosis hin, war allgemein. Bei Tieren mit intakten Puffernerven war 0.3 γ /kg weitaus überwiegend die minimale Wirkungs-dosis. Nach Durchschneidung der Puffernerven aber ergab sich, dass 0.1 γ /kg der Häufigkeit nach vorherrschte. Am häufigsten, und zwar in 8 Fällen, gab es eine Verschiebung der minimalen Wirkungs-dosis der gegebenen Injektionsreihe um eine Stelle zur kleineren Dosis hin. Von diesen waren in 7 Fällen eine Verschiebung von 0.3 zu 0.1 und in einem Falle von 0.1 auf 0.05 γ /kg. Bei den übrigen 5 Fällen umfasste die Verschiebung zwei und mehr Stellen. Von diesen betrug die Verschiebung in 2 Fällen von 0.6 auf 0.1, d. h. um zwei Stellen, in einem Falle von 0.6 auf 0.05, d. h. eine Verschiebung um drei Stellen und in 2 Fällen blieb die minimale Wirkungs-dosis bei erhaltenen Puffernerven im Bereich der gegebenen Dosierungen unerreicht, jedoch nach der Ausschaltung der Puffernerven ergab sich hier als minimale Wirkungs-dosis einmal 0.1 und das andere Mal 0.3 γ /kg. Die Blutdruckausschläge, meistens auch die der Ringerschen Lösung, zeigten im Gegensatz zur vorigen Gruppe nach Durchschneidung der Puffernerven eine Vergrößerung und wie aus der Tabelle ersichtlich, oft sogar eine recht beträchtliche.

Da bei den Versuchen dieser Serie die Vagi vor Beginn der Versuche durchschnitten waren, sind diese Befunde vaskulärer Herkunft.

II. Von der Einwirkung der Vagotomie auf die minimale Wirkungs-dosis.

Die Versuche dieser Serie wurden insgesamt an 9 Tieren vorgenommen. Die Blutdruckeffekte gleicher Dosierungen wie in Tab. 1, wurden vor und nach der Vagotomie mit einander verglichen. Beide Vagi wurden isoliert von den Depressoren am Halse durchgeschnitten.

Es ergab sich, dass, wenn diese Injektionsserie unmittelbar nach Durchschneidung der Vagi gemacht wurde, dann die Blutdruckeffekte sehr häufig noch mehr heruntergedrückt waren als vorher und die Maskierung der minimalen Wirkungs-dosis erwies sich als tiefergreifend. Wenn jedoch diese Injektionsserie nach einiger Zeit wiederholt wurde, verhielten sich die Blutdruckausschläge wie vor der Vagotomie. Aus diesem Grunde wurde in der Regel nach der Vagotomie 20 Min. gewartet und beim Vergleich der hierauf erhaltenen Ergebnisse mit denjenigen vor der Vago-

Tabelle 2.

Einfluss der Puffernerven auf die Höhe der Blutdruckeffekte einer steigenden Dosierungsserie von Adrenalin.

Versuche an vagotomierten Tieren.

Dosis in γ /kg.	A. Einer von 8 sich ähnlich verhaltenden Versuchen.					B. Einer von 4 sich ähnlich verhaltenden Versuchen.				
	Höhe des Blutdruckauschlages in mm Hg. bei		Vergrößerung d. Blutdruckauschlages in			Höhe des Blutdruckauschlages in mm Hg. bei		Vergrößerung d. Blutdruckauschlages in		
	intakten Puffernerven.	durchgeschn. Puffernerven.	mm Hg.	%		intakten Puffernerven.	durchgeschn. Puffernerven.	mm Hg.	%	
0.1	8	18	10	125		11	11	0	0	
0.3	9	20	11	122.2		14	13	—1	—7.1	
0.6	11	22	11	100		16	17	1	6.2	
1.0	—	—	—	—		21	22	1	4.7	
2.0	16	31	15	93.7		26	26	0	0	
3.5	22	41	19	86.3		31	33	2	6.4	
6.0	36	46	10	27.7		33	38	5	15.1	
8.0	40	48	8	20		35	42	7	20	
11.0	48	50	2	4.1		40	49	9	22.5	
15.0	52	52	0	0		44	47	3	6.8	
20.0	52	50	—2	—3.8		47	47	0	0	

tomie konnte keinerlei Wirkung auf die minimale Wirkungs-dosis festgestellt noch der Vagotomie zugesprochen werden.

Dieses Herabdrücken der Blutdruckeffekte gleich nach der Vagotomie kann nicht auf Rechnung des Durchschneidungsreizes der Vagi gesetzt werden, denn, wenn die Sinuse denerviert und die Depressoren durchschnitten waren, traten diese Erscheinungen in Verbindung mit der Vagotomie nicht mehr hervor.

III. Von der Wirkung der Vagotomie auf die Höhe der Blutdruckeffekte der grösseren Dosenbereiche.

Die Versuchsergebnisse dieser Serie wurden von 11 Tieren erhalten. Die Blutdruckeffekte gleicher Dosen, wie in Tab. 2, wurden vor und nach der Vagotomie verglichen. Die Resultate sind gleichartig. Eine Vergrößerung der Blutdruckeffekte konnte nicht konstatiert werden.

Die seit OLIVER und SCHÄFER (loc. cit.) gemachten Beobachtungen, dass sich nach der Vagotomie die Blutdruckeffekte des Adrenalins wegen Fortfalles der vagalen Herzhemmung vergrössern, ist mit den vorliegenden Funden nicht im Einklang. Da aber beim

Kaninchen der Vagustonus am schwächsten ist, verglichen mit Katzen und Hunden, an denen von anderen Autoren die entsprechende Beobachtung gemacht wurde, brauchen die Befunde der anderen Autoren durch die vorliegenden Feststellungen noch nicht kritisiert zu werden. Soweit es jedoch die Vagotomie betrifft, waren bei diesen Fällen die Depressoren zusammen mit den Vagi durchschnitten, wie sie bei Katzen und Hunden am Halse in den Vagi enthalten sind.

Bei 8 Tieren wurden die Vagi zusammen mit den Depressoren durchschnitten und die gleiche Injektionsserie gemacht wie oben angeführt. In 5 Fällen konnte keine Vergrößerung der Blutdruckeffekte nach Durchschneidung der Vago-depressoren konstatiert werden. Bei den übrigen 3 Fällen vergrößerten sich die Blutdruckeffekte nach Durchschneidung der Vago-depressoren mit Ausnahme der Blutdruckeffekte kleiner Dosierungen (bis zu $0.6 \gamma/\text{kg}$). Im restlichen Dosierungsbereich war die Vergrößerung entsprechend der grösseren Dosis auch grösser, bis von einer gewissen Dosis an, wovon im nächsten Abschnitt die Rede sein wird, die Vergrößerung der Blutdruckeffekte sich wieder zu verringern begannen. Diese Vergrößerung der Blutdruckeffekte ist vaskulär.

IV. Von der Wirkung der vasodepressorischen Reflexe auf die Höhe der Blutdruckeffekte des Adrenalins.

Die Höhen der Blutdruckeffekte der am vagotomierten Tiere vorgenommenen Injektionsserie wurden mit denjenigen verglichen, die nach Durchschneidung der Depressoren und Entnervung der Sinuse erhalten waren.

Im Einklang mit den Ergebnissen der minimalen Wirkungs-dosis stellte sich in einer Gruppe der Fälle (8) auch bei diesen Versuchen im Bereich der minimalen Wirkungs-dosis eine Vergrößerung ein. Von diesen 8 Fällen ist in Tab. 2 ein Beispiel unter A gebracht. Bei den übrigen 4 Fällen von denen ein Beispiel unter B gebracht ist, zeigten die Blutdruckeffekte kleiner Dosierungen kein Anwachsen. In Richtung der Vergrößerung der Dosierungen erschien jedoch in beiden Gruppen ein Anwachsen der Blutdruckeffekte, das sich verschieden fortsetzte.

Bei den obengenannten 8 Fällen ist charakteristisch, dass die Vergrößerung der Blutdruckausschläge — in absoluten Werten ausgedrückt — mit Vergrößerung der Dosis anwächst, während aber

Tabelle 3.

Verhalten der Vergrößerung der Blutdruckausschläge einer steigenden Dosierungsserie von Adrenalin in Verbindung mit Ausschaltung der Puffernerven von niedrigerem und höherem Niveau des Ausgangsblutdruckes. Ergebnisse stammen von einem und demselben Kaninchen, vorher vagotomiert.

Dosis in $\gamma/\text{kg.}$	Höhe des Blutdruck- ausschlages vom Aus- gangsblutdruck von 72 mm Hg.		Vergrös- serung d. Blutdruck- ausschlages in		Höhe des Blutdruck- ausschlages vom Aus- gangsblutdruck von 90 mm Hg.		Vergrös- serung d. Blutdruck- ausschlages in	
	vor	nach	mm Hg.	%	vor	nach	mm Hg.	%
	Ausschaltung d. Puffer- nerven.				Ausschaltung d. Puffer- nerven.			
0.1	10	10	0	0	4	12	8	200
0.3	13	13	0	0	5	14	9	180
0.6	18	19	1	5.5	12	23	11	91.6
1.0	23	25	2	8.7	17	27	10	58.8
1.5	28	32	4	14.2	22	34	12	54.5
2.0	30	37	7	23.3	25	38	13	52
3.0	35	45	10	28.5	30	44	14	46.6
4.0	38	50	12	31.5	33	46	13	39.3
5.0	39	53	14	35.8	36	49	13	36.1
6.0	42	57	15	35.7	38	50	12	31.5

gleichzeitig der prozentuale Zuwachs umgekehrt, sich mit Ansteigen der Dosis vermindert. Bei den übrigen 4 Fällen erscheint eine Vergrößerung der Blutdruckausschläge bei grösseren Dosierungen, wobei sich aber die absoluten wie auch die prozentualen Werte der Blutdruckausschläge mit dem Ansteigen der Dosis vergrössern.

Bei den beiden Gruppen der Fälle geschieht die Vergrößerung der Blutdruckeffekte in Verbindung mit dem Durchschneiden der Puffernerven nur bis zu einer gewissen Dosis, von welcher an beide, sowohl die absoluten wie auch die prozentualen Werte abzunehmen beginnen, bis wie bei Fällen, wo man bis zu genügend grossen Dosen gehen konnte, eine Vergrößerung der Blutdruckeffekte nicht mehr stattfindet.

Wie sind diese Befunde zu deuten?

Die Vergrößerung der Blutdruckausschläge in Verbindung mit dem Durchschneiden der Puffernerven drücken bei der gegebenen Dosis die Stärke oder das Mass des vasodepressorischen Reflexes aus. Der Zuwachs des Blutdruckausschlages bei der gegebenen Dosis ist Folge des sich auf den sino-aortalen Rezeptoren auswirkenden Blutdruckanstieges. Wir wissen, dass die prozentuale

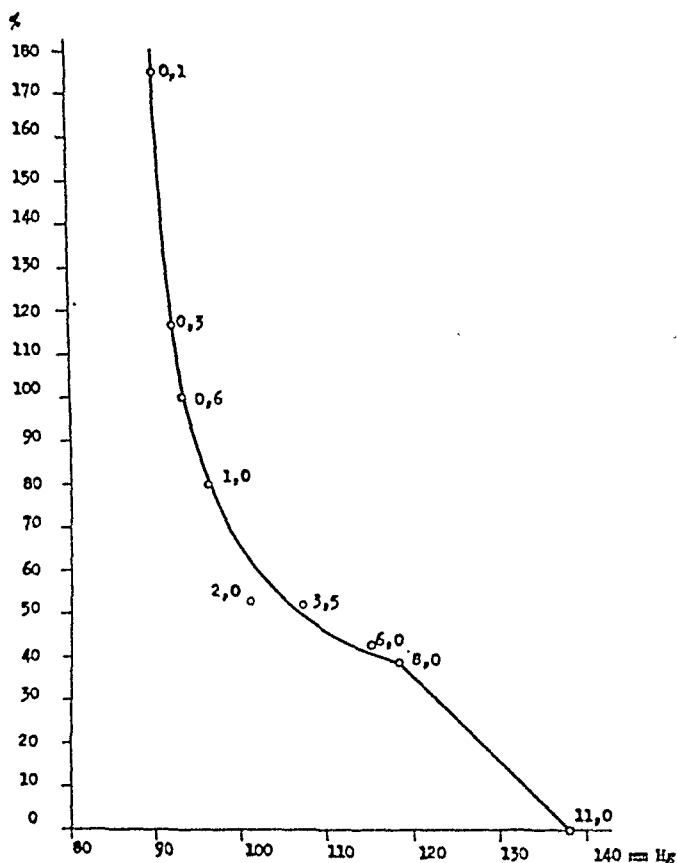


Abb. 2. Kaninchen 1.7 kg, vagotomiert.

Abszisse: Bei den intakten Puffernerven erhaltene absolute Höhen der Blutdrucksteigerungen (gemessen vom Nullstand des Manometers aus) einer steigenden Dosierungserie von Adrenalin. Die Zahlen an der Kurve sind Dosen in γ/kg .

Ordinate: Prozentuale Vergrößerung dieser Blutdruckeffekte infolge Durchschneidung der Puffernerven.

Grösse des vaskulären Reflexes den endosinualen Drucksteigerungen entsprechend im Koordinatensystem eingetragen, die bekannte Kurve mit sigmoidalem Verlauf ergibt (Koch usw.). Daraus ist ersichtlich, dass die Stärke des vasodepressorischen Reflexes absolut betrachtet, mit dem Wachsen des intrasinualen Druckes zunimmt, relativ gesehen aber ist die Effektivität des vasodepressorischen Reflexes beim normalen Blutdruckniveau am grössten. In Übereinstimmung hiermit vermindert sich bei der Gruppe, welcher der unter A gebrachte Fall entnommen ist, die prozentuale Vergrößerung der Blutdruckausschläge mit dem Steigen der Dosen, wogegen sich gleichzeitig das absolute Anwachsen ver-

grössert. Bei den sich dem des Abschnittes B analog verhaltenden Fällen stellten sich die vasodepressorischen Reflexe bei kleinen Dosen als inaktiv heraus. Es entspricht nicht den Erwartungen, dass im Bereich des normalen Blutdruckniveaus die vasodepressorischen Reflexe am aktivsten sein müssten. In Tab. 3 sind die Blutdruckeffekte einer Injektionsserie von demselben Tier, von höherem und niedrigerem Ausgangsblutdruck, vor und nach dem Durchschneiden der Puffernerven gebracht. Man sieht, dass sich die absoluten und prozentualen Werte des Anwachsens der Blutdruckeffekte beim höheren Stand des Ausgangsblutdruckes wie im Fall des Abschnittes A und beim niedrigeren Stand desselben, wie beim Fall des Abschnittes B der Tab. 2 verhalten. Das gestattet die Annahme, dass bei den Fällen der B-Gruppe der Ausgangsblutdruck des Tieres unter den Versuchsbedingungen niedriger lag als sein normaler. Zum Schluss noch über das erneute Vermindern des Anwachsens der Blutdruckausschläge. Wenn das Herabdrücken der Blutdruckeffekte des Adrenalins nur eine quantitative Antwort des Blutdruckanstieges auf die sino-aortalen Rezeptoren wäre, dann müsste das absolute Anwachsen der Blutdruckeffekte nach dem Durchschneiden der Puffernerven mit der Vergrösserung der Dosierungen so lange zunehmen, bis der Blutdruckausschlag der folgenden grösseren Dosis grösser wäre. Die Resultate der Experimente zeigen hier, dass sich die Vergrösserung der Blutdruckausschläge nach der Ausschaltung der Puffernerven nur bis zu einer gewissen Dosis an diese Beziehung hält, von wo an das Anwachsen der Blutdruckausschläge wieder abnimmt und zuletzt Null wird. Sowohl das Zu- und Abnehmen der Vergrösserung der Blutdruckeffekte, und der Übergang des einen Erscheinungsverlaufes in den anderen ist bei einigen Fällen fließender bei anderen abrupter. In einem kurvenmässig dargestellten Falle in Abb. 2, wo man die bei den intakten Puffernerven erhaltenen absoluten Höhen der Blutdrucksteigerungen (gemessen von Manometer Null an) einer steigenden Dosierungsserie als Werte der intrasinalen Druckanstiege gegenüber der nach Durchschneidung der Puffernerven erhaltenen prozentualen Vergrösserung der Blutdruckeffekte als Mass der Effektivität der vasodepressorischen Reflexe ausdrückt, sieht man den Verlauf der Kurve bei 8 γ /kg plötzlich abbrechen und die Effektivität des vasodepressorischen Reflexes Null werden. Bei anderen Fällen konnte das Wenden zur Abszisse fließender sein. Jetzt, wo der Verlauf der Kurve abbricht muss ein anderer Faktor den Verlauf ablenken, denn erwartungs-

gemäss sollte die Kurve bei Grösserwerden der Druckanstiege, die schon in der Richtung der Abszisse eingenommene Steilheit beibehalten. Dieser ist die Schwächung des Reflexerfolges, wie sie andere Autoren im grösseren Dosierungsbereich unter Adrenalinwirkung konstatiert haben. Mit anderen Worten, das Adrenalin in grossen Dosen greift zerstörend in die Verrichtung der Pufferreflexmechanismen ein und es wird also eine Frage der Dosierung sein, in welchem Umfange diese Reflexe wirksam bleiben.

Zusammenfassung.

1. Die nach der Höhe des Blutdruckausschlages im Blutdrucktest bestimmte minimale Wirkungs-dosis blieb sich bei intakten Puffernerven in 3 Fällen von 16 nach Durchschneidung der Puffernerven gleich. In diesen 3 Fällen war die minimale Wirkungs-dosis durch den Puffermechanismus nicht maskiert. Bei den übrigen 13 Fällen wurde die minimale Wirkungs-dosis durch den Puffermechanismus maskiert. Da bei diesen Versuchen die Vagi vor Beginn der Versuche durchgeschnitten waren, rühren diese Befunde von der vaskularen Gegenregulation her.

2. Die isolierte Vagotomie, bei der die Depressoren und die Sinusnerven intakt geblieben waren, übte keinerlei Einfluss auf die minimale Wirkungs-dosis noch die Höhe des Blutdruckausschlages in einem anderen Dosierungsbereich aus. Wenn die Vagi zusammen mit den Depressoren durchgeschnitten wurden, die Sinusnerven aber intakt blieben, konnte man in 5 von 8 Fällen keine Vergrösserung der Blutdruckausschläge konstatieren. Bei den übrigen 3 Fällen vergrösserten sich die Blutdrucksausschläge entsprechend der Dosisgrösse, mit Ausnahme der kleinen Dosierungen — bis 0.6 γ /kg, deren Blutdruckeffekte ihre frühere Höhe beibehielten.

3. Die manifesten Blutdruckeffekte des Adrenalins werden betreffs ihrer Höhe von den vasodepressorischen Reflexen herabgedrückt. Bei 8 von insgesamt 12 Fällen entsprach die Vergrösserung der Blutdruckeffekte der Relation der sich auf die Höhe des Blutdruckanstieges beziehenden Effektivität der vasodepressorischen Reflexe. Bei den restlichen 4 Fällen gab es keine Vergrösserung der Blutdruckeffekte kleiner Dosen, doch konnte diese anlässlich grösserer Dosierungen beobachtet werden. Hier trat diese bei 8 Fällen vorhandene Relation nicht in Erscheinung. Es kann angenommen werden, dass diese Verschiedenheit davon

herrührt, dass der Ausgangsblutdruck dieser Tiere unter den Versuchsbedingungen niedriger als normal war, denn in diesen 4 Fällen entsprechendes Verhalten konnte man beim gleichen Versuchstier mit niedrigerem Ausgangsblutdruck vorführen, wogegen man bei höherem Ausgangsblutdruck in den 8 Fällen entsprechendes Verhalten konstatieren konnte.

4. Die vasodepressorische Reflexfolge begann von einer gewissen grösseren Dosis an schwächer zu werden um zuletzt zu verschwinden. In grossen Dosen greift das Adrenalin zerstörend in die Verrichtung der Pufferreflexmechanismen ein und es wird eine Frage der Dosierung sein, in welchem Ausmasse diese Reflexe wirksam bleiben.

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The Occurrence of Citric Acid in the Human Thyroid Gland.

By

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1. Introduction.

The presence of Ci (= citric acid, free or in the form of citrate) in the thyroid gland of cattle, was shown in 1940 by HALLMAN in SIMOLAS' laboratory in Helsingfors. The mean value of his 10 determinations was 740 p. p. m. (p. p. m. = part pro million = microgram per gram) with the ultimate values of 295 and 1230.

At this time the common occurrence of citric acid in animal fluids and tissues was already known (see GEMMILL 1934, GRÖNVALL 1937, LENNÉR 1934, MÅRTENSSON 1940, PUCHER, SHERMAN and VICKERY 1936, SCHERSTÉN 1936 and THUNBERG 1933). It was therefore to be expected that Ci might also be found in other organs hitherto unexamined for this substance. Thus the significant point was not so much the actual discovery of Ci in the thyroid gland as the unusually high concentration of citric acid observed by HALLMAN.

HALLMAN has divided the tissues into a number of groups according to their Ci concentration. One group contains in addition to the thyroid gland the mammary glands, with a Ci value of about 3000 p. p. m. As milk is known to have a considerable Ci concentration the Ci value obtained might possibly be due to milk remaining in the gland. HALLMAN points out, however, that his results were obtained from mammary glands which had

been thoroughly rinsed several times and, therefore, the high values obtained could not be due to any remaining milk.

Ovaries and testicles compose a second group with a Ci concentration in fresh gland of 50—60 p. p. m. Another group showed the same Ci concentration as the blood in animals, *i. e.* between 15 and 20 p. p. m. To this group belong the brain, lungs, adrenal glands, spleen, kidneys, pancreas, parotid gland, and the thymus. — A group with a lower Ci value than that of the blood contains skeletal and heart muscles and the liver.

In this classification of organs and tissues after their Ci concentration the osseous tissue is lacking with its exceptionally high Ci concentration of up to 20 000 p. p. m. (2 %). DICKENS' discovery of this fact was, however, not yet available when HALLMAN made his above-mentioned analysis.

As pointed out above HALLMAN's Ci values of the thyroid gland are related to cattle. They cannot simply be applied to other animals and especially not to human material.

2. Collection of the thyroid material.

In the present investigation a study has been made of 75 thyroid glands in man. They were obtained in autopsies at the Pathological Institute of Lund during the winter 1945—1946. The material varies considerably as to the age and the disease of the individuals from whom it was obtained. The thyroid glands were collected only in the autopsies where one of us (B) was present. When examining and dividing the material the clinical journals were made use of as well as the autopsy findings.

The question arises whether the Ci content of the thyroid gland has undergone a change during the time between the death and the Ci analysis. This period has been of varying length. After death at least 5 hours elapsed before the post-mortem was performed. Sometimes this interval was as great as 24 hours as no post-mortems are made on Sundays. After the autopsy the gland was kept in a refrigerator for a few hours up to one or two days before analysis. The temperature in the refrigerator was not controlled. — The possibility of the Ci content having changed during this interval must of course be taken into consideration.

The Ci content may have been increased through the starting of autolytic processes, but it is also possible that the Ci content diminishes. In judging the probability of these two possibilities an observation by HALLMAN is of interest. As a result of experiments *in vitro* made on the thyroid gland in cattle, he states that isolated gland does not show the same Ci formation as is found in other tissues. The break-

down of Ci, too, was much slower in his experiment. — Our own experiments showed at least so much that post-mortal changes in the Ci content could not be excluded, but that, on the other hand, they did not assert themselves very strongly. A slight diminishing of the Ci content of the gland between the death and the analysis seems to be most likely, however. The size of other factors involved, however, makes positive results difficult. With the methods of investigation used the post mortal changes in the morphological structure of the glands did not have the time to assert themselves, and none have been observed in the microscopic examination.

The thyroid glands were dissected free from the outer capsule and the surrounding connective tissue and weighed. They were spread out in the shape of a butterfly and the outer parts, which usually contained the parathyroid glands, were cut away. Two sagittal sections were cut from the two side-lobes and the rest was left for Ci-determination. The microscopic preparations were made after paraffin embedding and were stained with hematoxylin and eosin. In some cases, where a few follicles were considerably above average size, pure colloid was taken for separate Ci analysis. For the same purpose calcareous concretions were taken in one case, and in two or more cases adenomas were examined separately, chemically and microscopically.

3. The method of determinating citric acid.

The citrate values submitted below were obtained with the colorimetric modification of the pentabromacetone method worked out by PUCHER, SHERMAN and VICKERY. Pyridin was scarce at the time of our investigation so that a substitute had to be found capable of stabilizing the yellow colour formed when sodium sulfide is added to pentabromacetone. Various substitutes have been suggested (acetone, glycerin and dioxane), but we ourselves have only used acetone. In later experiments, however, we have adopted the use of ethylene glycol as suggested by HUNTER and LELOIR (1945). We, too, found that this compound maintains the colour of the definitive solution better than does any other substitute, and moreover, produces a very high intensity of colour. As is now the rule, hydrogen peroxide was used in the decoloration of the surplus of potassium permanganate. Lately, however, we have used sodium nitrite according to HUNTER and LELOIR.

4. The citric acid concentration in the human thyroid gland.

In the quantitative registration of the general occurrence of citric acid in the human thyroid gland, it has been considered most suitable to sort out the undeveloped glands of individuals less than 1 year old. After this elimination there remained 58

glands as a basis for numerical calculations. The mean value of the citric acid concentration was 890 p. p. m. and the standard deviation 420. This figure is of about the same order of magnitude as HALLMAN's determination in cattle (730 p. p. m.). No significance can be attached to the difference on account of the difference in the origin of the species.

The values obtained of the citric acid concentration in the thyroid gland can be classified in various groups and on different basis according to the questions under investigation. This report, however, will only illustrate the dependence of the citric acid concentration on such elementary factors as the morphological structure of the gland, the age and sex of the individuals, and pathological changes in the organism. The material is divided with this aim in view. When more specific problems are studied, the material must naturally be divided accordingly. Some questions may require animal tests.

5. The influence of the morphology of the gland.

Differences in the morphological structure of the thyroid gland are most varied and probably provide the best basis for a classification of the material. The obvious dissimilarity of structure between the glandular epithelium and the colloid brings up the question of the distribution of the citric acid in these two components. Variations in the structure of the gland are of interest also in so far as a more or less active follicular epithelium and different kinds of goiter are capable of affecting the citric acid concentration of the gland. These problems will also be discussed in the following within the range of the material.

On account of the experience of one of us (B) of the advantages of quantitative micromorphological analysis, an application of this method was considered. However, as the present material represents thyroid glands with extreme variations of structure and as an exact quantitative analysis would be very slow work, it was considered sufficient to make the classification by comparing the microscopic preparations. A division into four groups has been made according to the colloid content of the glands. First those preparations were sorted out that contained a sparse quantity of colloid (Group I). Subsequently those preparations were brought together in which the colloid was extremely ample

(Group IV). Between these two groups it was possible to insert two more groups, one with a moderate amount of colloid (Group II) and the other with abundant colloid (Group III). If the preparations within the four different groups are arranged according to their rising colloid content the boundaries between the groups will not be distinct, although the preparations representing the middle of one group are clearly different from those representing the middle of the adjoining groups, as illustrated by Figs. 1—4.

The average content of citric acid of each of the four groups is expressed in milligram per gram of glandular tissue. The result is graphically illustrated in Fig. 5, which figure also shows the number of glands in each group.

It appears that the quantity of citric acid increases strongly as the colloid is augmented. In three cases the colloid was collected for separate determination of the citric acid. In one case, a gland of average size and of otherwise essentially normal appearance a few small nodose parts were found, from which pure colloid was obtained. The citric acid concentration in the gland was 0.50 mg. and in the colloid 0.69 mg. per gram. Another case was a large nodose colloid-rich goiter, from which both brownish-yellow, viscous colloid and ordinary, lighter and less viscous colloid was taken for examination. The citric acid concentration was 0.68 mg. per gram in the atypic colloid respectively 1.34 in ordinary-looking colloid, while the usual test from the gland showed a value of 0.84 mg. The third case was an average-sized pale gland, uncommonly rich in colloid and containing some follicles the size of peppercorns, from which colloid was collected. The pure colloid held 1.08 and the remaining part of the gland 0.97 mg. per gram.

As has been mentioned above there is a direct relationship between the colloid and citric acid contents so that glands with ample colloid have a high concentration of citric acid and glands with very little colloid a relatively low one. When, moreover, a high citric acid concentration was observed in the isolated colloid the conclusions seems justified that *the large amount of citric acid in the human thyroid gland is to be found chiefly in the colloid.*

As the metabolic processes probably take place in the epithelial cells of the follicle, and citric acid being an important product in the intermediary metabolism, it would be of great interest to know the behaviour of the citric acid content of these cells, especi-

ally as the metabolism of the thyroid gland is as a rule rather high. The large amount of citric acid in the colloid, however, makes an investigation of this kind considerably more complicated. In this connexion it may be mentioned that the glands apparently containing a very small amount of colloid had a low concentration of citric acid when compared to other glands, but that this value in itself could be considered to be high. No gland showed a lower concentration than 0.07 mg. of citric acid per gram of glandular tissue. In a few glands from newborn or premature children, the colloid in the examined preparations was so sparse that several fields of vision had to be examined to find follicles containing colloid. This small amount of colloid could hardly explain the actual citric acid quantity. — This may indicate that the epithelial cells of the follicles are also rich in citric acid, although to a lesser degree than the colloid. It is impossible, however, to discard altogether the possibility that the citric acid quantity of the above-mentioned glands may depend on colloid, as the presence of colloid was not investigated in the whole gland and as the formation of colloid begins rather early in fetal life. However, an investigation by ORATOR and SCHLEUSSING (1931) speaks in favour of the possibility of the glands' being free from colloid. They had found several glands from the first month of life where no colloid could be found (and no iodine either).

Various functional conditions of the epithelial cells of the follicles, as shown by microscopic examination, can affect the citric acid concentration. Thus the colloid quantity is rather large in an inactive gland and, consequently, the citric acid concentration high. In the present material this fact has proved to be valid both for normal variants and for cases which may be classified as colloid goiters.

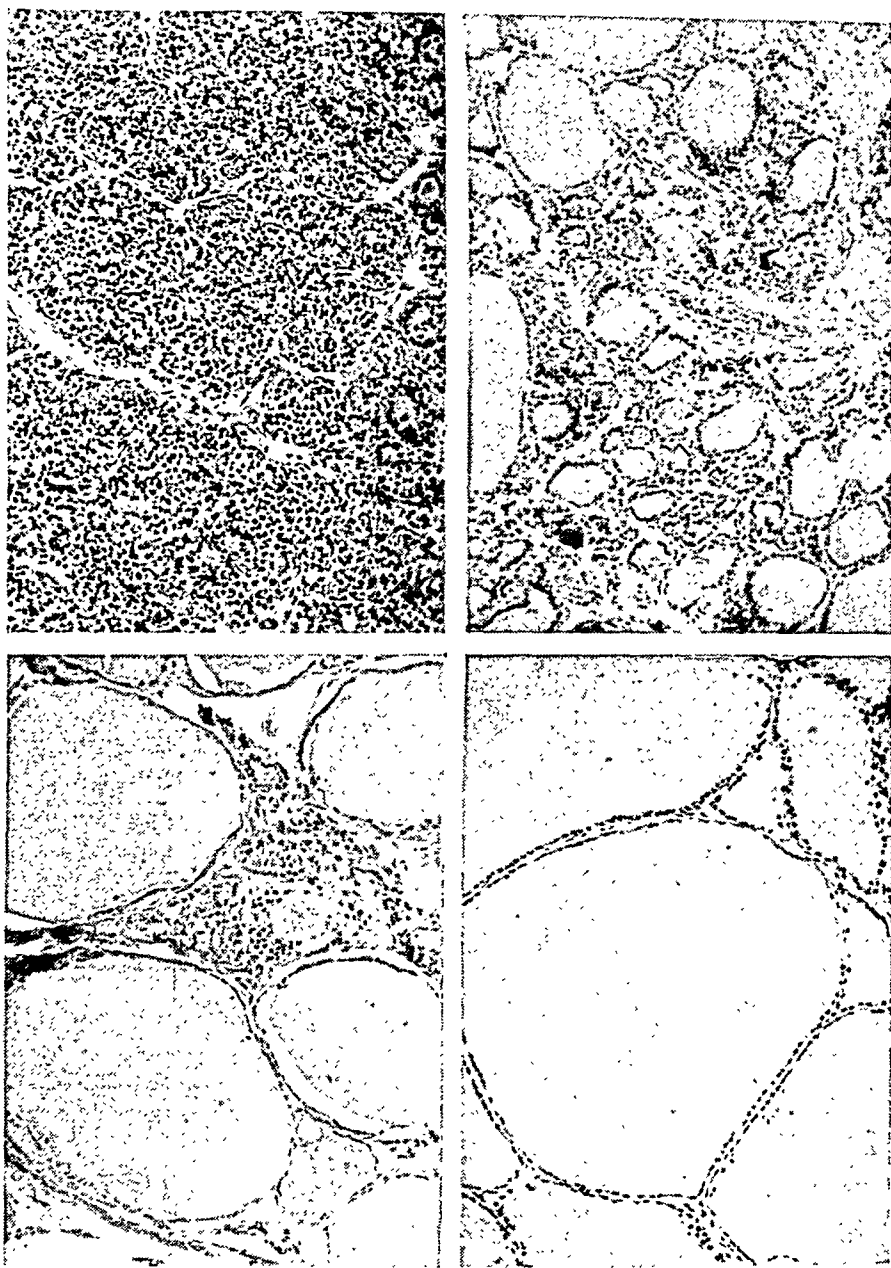
In a case of basedowified goiter with very sparse colloid a particularly active highly proliferating follicular epithelium was observed. Treatment with Lugol's solution had only been given during the last two days of life so that no effect would probably have had time to manifest itself. The citric acid concentration was low or 0.10 mg. per gram of gland. In two cases parenchymatous adenomas with sparse colloid were examined. In one case the Ci-concentration of the adenoma was 0.56 mg. and in the gland 1.62 mg. per gram. The corresponding values in the other case were 0.40 and 1.30. The citric acid quantity in adenomas is not larger than can be explained in these cases by the sparse colloid

amount. It is possible, on the other hand, that the active epithelium contains citric acid, though not in such a degree as the colloid.

6. The influence of age on the Ci-concentration.

In an investigation of the presence of citric acid in different age groups the results will be considerably affected by variations in the colloid quantity. The inconsiderable amount of colloid in the newborn and the increase thereof the following years, for instance, can influence the citric acid values. Not only the amount of colloid in different ages of life is important but sometimes also the quality. The colloid may contain salts like calcium and magnesium as shown in the human thyroid gland by micro-incineration by UOTILA and JÄÄSKELAINEN (1938) and by MELTZER and HEUSER (1939). Highly insoluble complex salts exist, which contain calcium, magnesium and citric acid. It is possible, therefore, that with increasing age citric acid becomes deposited as such insoluble salts, and so ceases to play any rôle in the metabolism of the gland. This possibility is stressed by the fact that a concrement, which was found in a nodose colloid goiter in a 78 year-old woman, contained a large quantity of citric acid, *i. e.* 6.30 mg. per gram. A high concentration of citric acid in concrements *i. a.* from thyroid glands was found by MÅRTENSSON (1941). Thus an investigation would be of interest to determine whether the citric acid concentration in older subjects reaches values so high that they cannot be explained by the existing quantity of colloid alone.

The material has been divided into different groups with a 10-year interval. The subjects who have died during the first year of life form a special age class. Within these age classes the mean value of citric acid per gram of glandular tissue has been calculated and the results brought together in Fig. 6. At first the citric acid content increases strongly and afterwards more slowly up till 25—30 years of age. Then there is a reduction which is moderate and uncertain at first but later becomes pronounced. With increasing age the citric acid content augments again slowly. As the citric acid concentration in old age does not reach higher values than several decades earlier, the investigation does not support the assumption that citric acid in the course of time becomes deposited in the form of insoluble salts.



Figs. 1—4. Microphotos of thyroid glands representing four groups with different colloid content. The amount of colloid is in Group I inconsiderable to sparse, in Group II moderate, in Group III rich, and in Group IV extremely rich.

The reduced concentration of colloid in the two age groups 51—60 and 61—70 could be the cause of the low citric acid values observed there. In order to ascertain whether the colloid

Table 1.

The citric acid content within various age classes and the distribution of the glands between the four colloid groups of the two sexes.

Age	Citric acid mg. per gram gland	♂				♀			
		I	II	III	IV	I	II	III	IV
0—10.....	0.13	11	1	—	—	7	1	—	—
11—30.....	1.01	—	—	1	2	—	—	—	2
31—50.....	0.99	—	—	7	1	—	—	5	—
51—70.....	0.65	1	3	3	2	2	3	4	2
71—90.....	1.01	—	2	6	3	1	1	3	1

content is lower in these two groups, they were put together into one and compared with other age groups with an interval of 20 years. The interval in the youngest age group is then only 10 years. Within these age groups the frequency of the various colloid groups in males and females has been noted and the results given in Tab. 1. This table shows that in both sexes the glands with low colloid concentration are to be found most frequently in the year groups 0—10 and 51—70. With regard to the second group this fact may be due to a real reduction of the colloid quantity during this period or to a distribution at random. The results of previous investigations confirm the first alternative. According to unanimous reports in the literature a reduction of the size of the follicle actually takes place in early middle life. (See inter alia MAY (1928), SCHÆR (1928), DE OCA (1930), ORATOR and SCHLEUSSING (1931), THOMAS (1934) and SAKA (1938). Various information is given of when this reduction begins (25—45 years of age) and it is not impossible that it sets in later in our Swedish population material.

On the basis of measurements SCHÆR has illustrated graphically the mean values of the follicle diameters for various ages. His curves are included in Fig. 6 and a fall of the citric acid values can be observed when the reduction of the follicle size has had time to assert itself. But the size of the follicle does not make an adequate measure of the colloid content. During the decrease in the follicular size the cells of the follicular epithelium must be assumed to be active, thus resulting in a lower concentration of colloid. When the cell has ceased to function the size of the cells of the follicular epithelium will be reduced and the gland will consist of small, closely stratified follicles with thin walls. In a gland of this type the colloid concentration will be high and

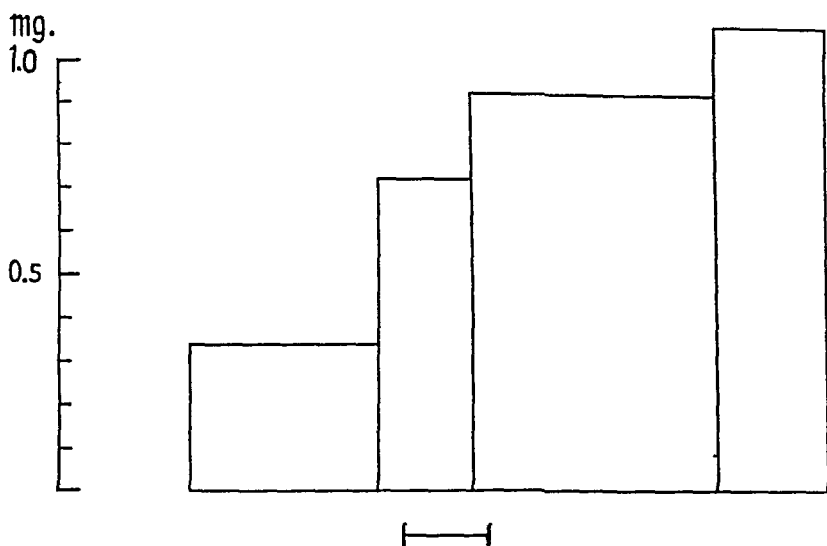


Fig. 5. Diagram showing the citric acid content of four groups of thyroid glands with different colloid content. The farthest to the left represent the glands with the lowest colloid concentration, which increases in the following groups. The height of the figures denotes the average amount of citric acid within the group expressed in mg. per gram of glandular tissue. The breadth of the figures corresponds to the number of individuals in the groups and the scale line below the figure indicates the number of 10.

changes of this kind may explain the increase of the citric acid values in advanced ages. The connexion between citric acid quantity and follicular size can perhaps also be expressed so that the mobilizing of colloid increases the cellular work and thus causes a greater consumption of citric acid in the metabolic processes.

According to what has been said above, it has only been possible to show such variation of age in the citric acid concentration as are connected with the presence of colloid. Thus there is no corroboration of the presumption that richness in colloid and citric acid concentration vary with the age and independently of each other. The distribution of the ages within the four colloid groups speaks against this presumption, too. Age is certainly low in Group I where several glands from individuals are undeveloped and poor in colloid. From Group II to Group IV, however, the increased citric acid concentration does not correspond with the increase in age. The average age is 54 years in Group II, 57 in Group III and 53 in Group IV.

As has been mentioned in the introduction the citric acid concentration of the thyroid gland of the two sexes is also of interest.

Table 2.

Group of disease	Citric acid content average mg. per gr gland			Age, average	Number of cases
	Actual value	Calculated values determined from the colloid content	Calculated value based on the age distribution		
Infectious diseases .	0.82	0.87	0.90	62	12
Diseases of the heart and vessels	0.71	0.88	0.76	50	9
Diseases of the heart and vessels complicated by other disease	0.97	0.89	0.82	63	8
Malignant tumours.	0.88	0.84	0.80	58	9
Malignant tumours complicated by other disease	1.26	0.95	0.85	69	6
Traumata	0.82	0.83	0.90	32	4
Diabetes complicated by other disease	0.81	0.97	0.97	61	5
Diseases that cannot be included in any of these groups . .	0.67	0.88	0.85	35	5
Premature infants .	0.34	0.34	0.38	3 d.	5
Asphyxia and congenital vitia	0.38	0.34	0.38	17 d.	5
Diseases of infancy (mostly infections)	0.40	0.45	0.38	120 d.	7

The average values of the citric acid concentration in thyroid glands from various groups of diseases. By way of comparison those citric acid values have been determined which were to be expected on the bases of the occurrence of colloid and the age distribution within the groups.

Before discussing this subject, however, it would be better to consider the effect of various morbid conditions.

7. The citric acid concentration in various morbid conditions.

When classifying the material in different groups according to the disease, that disease has been decisive that has affected the individual most strongly. The classification was based upon the findings in the autopsy and on the clinical history as stated in the hospital journals. In spite of this schematic differentiation it has not been possible to obtain homogeneous groups. Therefore,

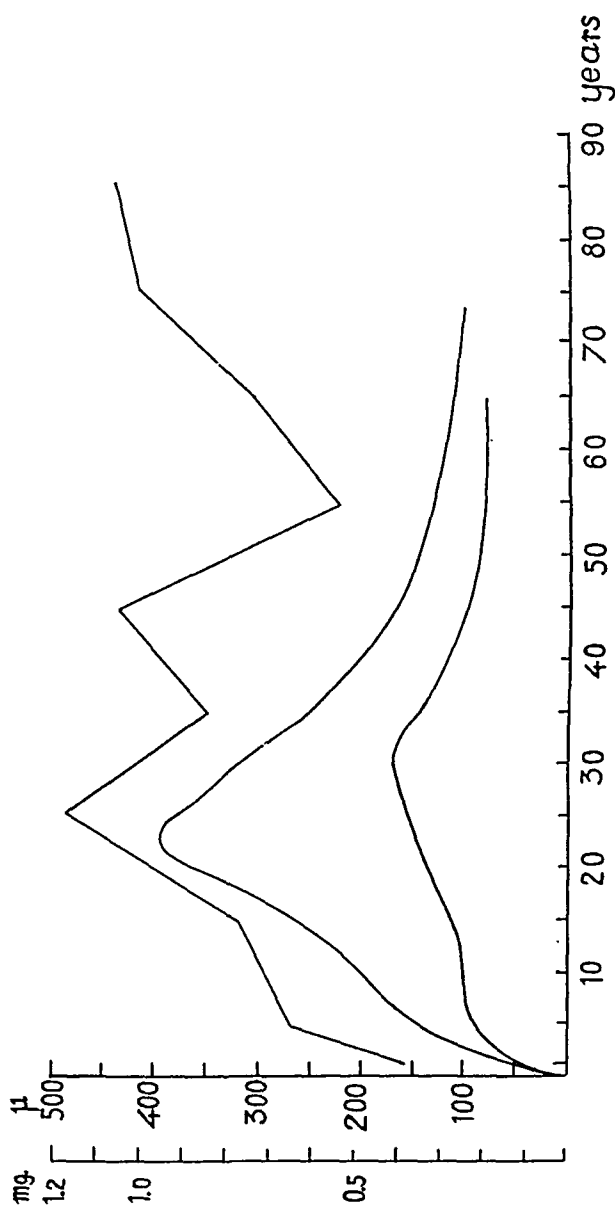


Fig. 6. The upper curve, corresponding to the scale farthest to the left states the citric acid concentration per gram of gland of the various age groups. The points of the curve do not represent isolated observations but the mean of the age classes. The two lower curves are taken from an investigation by SCHÄER and show the average diameter of the thyroid follicles at various ages from two districts of Germany.

when diseases of the heart and vessels or malignant tumours have been complicated by other disease these cases have been brought together into special groups.

As thyroid glands in the first year of life show essential differences both in structure and Ci-concentration compared to older glands, these young specimens were not included in the other groups but were classified by themselves.

In Table 2 the various groups of diseases have been brought together and the number of individuals within every group, their average age and the mean content of citric acid per gram gland, are recorded. The various groups consist of infectious diseases, diseases of the heart and vessels, malignant tumours, traumata, diseases of the heart and vessels complicated by other diseases, and malignant tumours also complicated by other disease. The complicating factor in the first group was mainly infections and in the second infections and diseases of the heart and vessels. In two of the four cases of the traumata group infections had set in. Separate groups were formed by five diabetic cases complicated by other disease (in 4 cases renal troubles), and by five cases that could not be included in any of the above-mentioned groups.

When comparing the various groups it is evident that the citric acid concentration of the thyroid gland varies very little. Only the group malignant tumours complicated by other disease showed a marked difference in citric acid value compared with the other groups. This does not seem to be due to any direct effect on the citric acid content of the thyroid gland but to the colloid rich glands that obviously dominate this group. These morbid conditions may cause a high content of colloid in the gland but, on the other hand, these findings may be purely accidental.

In studying the effect of the morbid conditions on the citric acid content it is not necessary to compare only the values of the various groups of diseases. Within each group an expected theoretical Ci-value can be calculated from the distribution among the four colloidal groups and serve as a basis for comparison. Another value is calculated from the distribution of age within the group of illness in question. The source of error is thus neglected of the individuals of this group affecting the calculated theoretical values. In Table 2 these theoretical values are presented. It will be noticed that no great discrepancy exists between the observed values and the calculated ones with the exception of the previously mentioned high value of the small group of malignant tumours complicated by other disease.

As mentioned before there was a special classification of the material obtained from individuals under one year of age. Three groups were distinguished. 1. premature infants 2. asphyxia together with congenital vitia and 3. other diseases of infancy

consisting mostly of infections. In this order a moderate increase in the citric acid values takes place, possibly due to the increase of the citric acid concentration with increasing age and the resulting increased presence of colloid. Consequently if these variations are not purely accidental they may be explained in this manner and cannot be attributed to the effect of the morbid condition in question.

As the citric acid content of the thyroid gland varies with the morphology of the gland, diseases causing changes in the structure are able to affect the citric acid concentration. Moreover, there is the possibility of the citric acid concentration changing independently of the structure. This possibility is not corroborated by this investigation, no great changes of this kind having been observed. Small changes can, if they exist, probably be established only by help of a considerably larger material.

As the various morbid processes do not seem to have any definite effect on the Ci-content of the thyroid gland, it is permissible to divide the material to such an extent that the occurrence of citric acid in the two sexes can be compared in the various age groups.

8. The influence of the sex.

In Table 3 the citric acid values in male and female individuals are reported for various ages together with data concerning the number and the average age of each group. With an arbitrary approximation the age of 15 to 45 years can be considered as fertile and the endocrine activity of the gonades during this period as most intensive. No sexual difference is observed, the citric acid value in men being 0.96 mg. per gram glandular tissue and in women 0.99 mg. However, there is a deviation in the values obtained from boys and girls with 0.31 and 0.59 mg. respectively.

No certain conclusions can be drawn, however, as most individuals of this age group represent newborn and infants. The prevalent large mortality in the boys is thus probably connected with the delayed development displayed by many of them. Then there is also the possibility that the thyroid glands of the boys in many cases are less developed than the girls' and consequently less rich in citric acid.

A remarkable difference appears during the 30-year period after the age of 45, when the citric acid of the men is still about

Table 3.

The Ci-content of the thyroid gland in the two sexes within various age groups.

Age group	Average Ci-value mg. per gram gland		Number of individuals		Average age	
	♂	♀	♂	♀	♂	♀
0—14.....	0.31	0.59	12	8	—	—
15—45.....	0.96	0.99	8	7	34	36
46—65.....	0.97	0.47	10	9	56	60
66—86.....	1.07	0.72	13	8	77	75

0.97 mg. per gm glandular tissue while the women's is but 0.47 mg., *i. e.* scarcely half. On an examination of these values the question arises if they are caused by chance. Through statistical analysis by help of the t-distribution it is possible even in small series to decide the degree of probability of their being random samples of the same mother population and thus, if discrepancies between them are accidental. The result of the calculations shows the value of *t* to be 2.79 which indicates with 98.5 per cent probability that the difference is not accidental. These calculations were made after the excluding of a case of basedowified goiter with a low citric acid value. It is remarkable that the change of the citric acid value occurs in the women but that no change has been observed in the men (Table 3). The extinction of the function of the gonades after the menopause can therefore be of special significance. The difference should then be due to hormonal factors. Since the morphology of the thyroid gland is especially sensitive to their influence, observations of changes in the glandular structure are significant. A comparison of microscopic preparations from men and women from 46 to 65 years of age does not suggest the existence of any difference in the colloid content. Nor have any sexual differences been noted in previous investigations submitted in this report, where the size of the follicles was registered for various ages. Thus there are in this material individuals of both sexes, who at the age of 51 to 70 years have a low colloid concentration (Table 1). There is no grounds for the assumption that the differences in the citric acid values of the two sexes are due to the changes in the structure, although their influence cannot be ignored altogether. Nor does any difference in the structure of the glands have any effect on their weight.

In the group 46 to 65 years the thyroid gland weighs 18.3 gm. in men and 18.6 gm. in women; the basedowified goiter mentioned before is not included in these figures.

The differences observed must therefore very probable be due to a more direct influence of the sex.

9. Citric acid concentration and occurrence of iodine.

From a comparative point of view variations in the iodine content of the thyroid gland is of considerable interest. In investigations of recent years the occurrence of iodine as thyroxine-iodine as distinguished from other iodine compounds, the differences between the two sexes at various ages do not seem to have been considered. Therefore a comparison between the observed citric acid content of the thyroid gland and the reports in the literature on the iodine content of the gland must be confined only to the general occurrence of iodine. Such an examination of the iodine in thyroid glands obtained in routine autopsies seems to have been performed last by KOLNITZ and REMINGTON (1933). They found that the total quantity of iodine in the gland showed a slow decrease from middle life on. The relative quantity of iodine, however, the object of the investigation in question, remained practically constant. Among earlier publications ZUNZ' research (1919) should be mentioned on the occurrence of iodine in individuals who have died in war after wound injuries. ZUNZ reports there a considerable variation without entering into any explanations of his results.

A comprehensive investigation of the occurrence of iodine in a Swedish post-mortem material has been carried out by JOLIN (1906). In his report he mentions no variations of interest in this connexion. The detailed tabular material has furnished us with the values from 29 glands of men and 21 glands of women at the age of 46—65 years which have been subjected to statistical study. These values give the quantity of iodine, expressed in mg. per gm dried glandular substance. The values show that glands from men contain 1.67 ± 0.14 and from women 1.57 ± 0.25 mg. iodine wherefore no difference exists during this period. As far as this question can be settled at present, the chemical structure of the gland does not show any correspondence to the observed sexual difference in the citric acid content. An explanation to

the highly probably existing sexual difference in the present material can therefore not be supplied.

It may be mentioned that several morbid conditions with a predilection for the age in question show a varying morbidity rate in the two sexes. Differences in the metabolic conditions may be one of the causes to be taken into account and among the great number of significant metabolites citric acid should also be observed.

10. Discussion.

The results of a very large number of determinations indicate that the mean value of the citric acid concentration in the blood serum of man is roundly 25 p. p. m. The citrate value of about 900 p. p. m. shown by the thyroid gland of man according to investigations submitted above, is thus of a very much greater order of magnitude. The question then arises which conditions cause this high citric acid concentration in the glandular tissue.

Naturally we are here concerned with a dynamic equilibrium between factors favouring the increased formation of citric acid and others acting in the opposite direction.

In view of these findings it is reasonable to assume that the thyroid gland produces more citric acid per time unit than organs with lower concentrations of citric acid. HALLMAN's observations, however, contradicts this assumption, at least his *in vitro* experiments show that the Ci-formation in the thyroid gland is low, even lower than in most organs.

Then there is the possibility that the citric acid is bound in the gland in some way or is prevented from free diffusion to the capillaries or the lymphatic vessels.

It is now generally agreed that part of the citric acid in biological fluids and tissues exists in a complex form, in combination with calcium and perhaps also with magnesium. Citric acid, therefore, should exist not only as citrate ions but also in the form of "calcitrate ions".

New views on the binding capacity of citric acid have recently been submitted by KUYPER (1945). While citric acid was previously looked upon as a calcium-dissolving agent, KUYPER showed that citric acid in combination with phosphoric acid can cause precipitation of calcium in animal fluids. In other words citrate is precipitated in the presence of calcium- or phosphate

ions, a precipitation that becomes quantitative when the proportions of the reacting substances and the proper pH are observed. In this manner part of the citric acid in the tissues is deprived of its power of diffusion.

It seems to be this process, described by KUYPER, that brings about the formation of calcium concrements containing high amounts of Ci such as have been reported especially by MÅRTENSSON in the thyroid gland.

In interpreting the high citric acid values of the thyroid gland, the possibility must also be considered of an accumulation of citrate within the cells in connexion with a possible intracellular formation of this substance. The structure of the citric acid molecule indicates a low capacity of penetration. A complex of three carboxyl groups and one hydroxyl group should make a diffusion out through the surface layer of the cells more difficult.

Naturally, there is also the question to what degree the colloid in the thyroid gland assists in forming and maintaining the high citric acid concentration in the gland.

However an explanation of the conditions responsible for the high Ci-concentration of the thyroid gland does not reveal the physiological significance of the citric acid in the thyroid gland. If, for instance, the thyroid gland is characterized as a Ci-depot, this will only lead to the physiological question: how is this depot filled and emptied? Do nervous or hormonal factors play any rôle here? And so on.

The amount of Ci in the thyroid as compared with the Ci content of the blood can be calculated as follows: If we estimate the amount of serum in man at 3 liters and assume the Ci-value to be 25 mg. per liter, we obtain a value of 75 mg. for the total concentration of citric acid in the blood. Assuming further an average weight of 20 grams for the thyroid gland of adult man and a citric acid content of 0.9 mg. per gram thyroid gland, the total Ci-amount of the thyroid gland will be 18 mg., *i. e.* one fourth of the circulating citric acid.

Summary.

The investigation is based on a material of 75 thyroid glands of man which have been obtained in routine autopsies.

1. On the basis of this material the general occurrence of the citric acid in the human thyroid gland was established and the concentration thereof quantitatively determined. The mean value of the citric acid concentration was found to be 890 p. p. m. In the determination of this value the undeveloped and rather Ciscarce glands of subjects less than one year old have been excluded. The standard deviation is 420 p. p. m. Compared to the blood and most tissues the citric acid concentration of the human thyroid gland is remarkably high, amounting almost to 1 mg. per gram of glandular tissue.

2. If the gland-material investigated is classified after the colloid content the citric acid has been found to increase greatly with the increase in the colloid content. The greater part of the citric acid is to be found in the colloid. In comparison with the colloid the concentration of citric acid in the follicular epithelium is low but otherwise no certain conclusions could be drawn. Some observations, however, suggest that the citric acid concentration in the epithelial cells may be considerably higher than in the blood.

3. The citric acid concentration in the thyroid gland increases greatly at first, and then more slowly up to the age of 25—30 years. After that period there is pronounced decrease, moderate and uncertain at first but gradually pronounced. With increasing age the citric acid increases again. This variation in the citric acid concentration agrees remarkably well with the structural changes of the age in question.

4. No variations in the citric acid concentration of the thyroid gland were shown to be primarily caused by disease. A secondary influence, however, has been observed owing to a change of the colloid content of the gland.

5. During the fertile ages of 15—45 years the citric acid concentration has been found identical in the two sexes. A sexual difference becomes evident, the female glands showing a considerably lower value during the subsequent 20-year period. The probability that this difference is not accidental, has been calculated to 98.5 per cent. No corresponding difference in the structure of the thyroid gland during this period has been observed previously, and none has been evidenced in our material. No iodine determinations were carried out, but statistical calculation on the basis of data given in previous reports in the literature show no sexual differences in the age concerned.

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On the Biological Assay of Gastrin.

By

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The investigations of KOMAROV (1938, 1942) and of UVNÄS (1942) have demonstrated that a protein-like substance, free of histamine, gastrin, exerting a strong secretagogue effect on the fundus glands of the stomach of cats and dogs can be extracted from the pyloric mucosa of different animals, pigs, dogs and cats. It does not arise during the extraction procedure which includes boiling of the mucosa with dilute hydrochloric acid. UVNÄS' experiments of 1942 show convincingly that the hydrochloric acid secretion of the fundus glands is at least in part regulated by a humoral mechanism located in the pyloric part of the stomach.

Both KOMAROV and UVNÄS gave fairly complete data about the preparation and the chemical properties of the gastrin. The details about their method of assay were not complete. Since, during our trials to prepare gastrin, we had to use a considerable number of cats for assaying our preparations, we have been able to supplement in some respects the statements of the previous authors.

The method of assay.

Like the previous authors, we have used cats, weighing on an average 3 (2—5) kg. They were starved 36 hours before the experiment.

The form of anaesthesia and the technique of operation on the

stomach were those of UVNÄS (1942). During a light ether anaesthesia 5 ml per kg of a solution containing 10 per cent of urethane and 1 per cent of chloralose were injected intravenously during the course of 10 minutes. The mixture of chloralose and urethane gave more consistent results than the use of chloralose alone.

The gastrin was prepared with the same technique as used by KOMAROV and by UVNÄS. The purest preparations contained a secretory unit per 2—3 mg. They were free of histamine as shown in tests on the guinea pig's small intestine.

The substance was dissolved in 20 ml slightly acidulated physiological saline solution and allowed to run into the vein (v. femoralis) of the cat from a burette during the course of 20 minutes, followed by a few ml of the saline solution. One hour was allowed to elapse after finishing the operation on the stomach before the first injection of gastrin was made. The amount of gastric juice secreted was collected at hourly intervals, the volume measured and the acidity titrated with 1/10 N sodium hydroxide, methyl red being used as indicator.

KOMAROV expressed the strength of his preparation in secretory units (S. U.), one S. U. being defined by him as »the quantity of extract sufficient to produce a secretion of 1 ml of gastric juice (1/10 N HCl) in one hour in a cat under chloralose-urethane anaesthesia». Applying the same principle as that used by WILANDER and ÅGREN (1933) in assaying secretin, we prefer to use the acidity of the juice expressed in tenth normal acid rather than the volume, as a measure of the gastrin effect. The total acidity more often corresponded to the amount of gastrin administered than the volume of the juice. The acidity was often somewhat stronger than tenth normal.

The secretory response to a dose of gastrin.

In assaying gastrin, full proportionality between the dose of gastrin administered and the amount of acid excreted is to be expected only within a narrow range of dosage. The dose can, however, be doubled, twice the amount of acid still being obtained. Beyond that range, the figures are less reliable. Smaller volumes of juice than 4—5 ml cannot be dealt with and volumes, exceeding 10—12 ml cause exhaustion of the secretory mechanism. Within these limits, however, a good proportionality can be obtained in one and the same cat, as is evident from table I.

Table I.

The secretory response to doubled doses of gastrin.

Cat	Weight in kg	Gastrin in mg	Gastric Juice in ml	HCl 1/10 n ml	Number in the series of the injection
122	4.1	30	5.75	4.00	7
		60	10.50	10.75	8
		120	12.00	13.00	9
123	2.9	50	5.00	4.50	3
		100	5.50	6.30	4
124	2.9	60	3.25	3.70	4
		120	7.00	8.05	5
126	3.5	50	5.75	6.50	8
		100	9.00	11.75	9
127	2.5	50	5.00	5.00	8
		100	8.00	9.40	9
129	2.0	20	6.80	5.60	1
		40	6.50	6.20	2
		80	9.50	11.80	3
125	3.5	30	5.00	5.20	3
		60	4.50	3.50	4
		120	5.00	4.50	5
128	2.9	30	4.00		6
		60	5.00		7

There are cats, however, which for one reason or another do not show this proportionality (125 and 128).

110 of the experiments were evaluated with this detail in mind. One and the same cat had been given two or three different amounts of gastrin, the dose usually being doubled in consecutive injections. Either there was satisfactory proportionality (+) or none at all (—) or a certain, although less satisfactory (\pm) response. The last happened mostly when some previous dose had been too large, causing an exhaustion of the secretory mechanism.

Table II.

The relationship between the amount of gastrin administered and the secretory response.

	Number of cats	+	—	\pm
A	57	33	14	10
B	53	34	10	9
	110	67	24	19

Full proportionality +; no proportionality —; less satisfactory response \pm .

In one of the series (A) 10 different samples of gastrin with a secretory unit in 5 to 60 mg were used and in the other series

(B) all the experiments were made with one and the same highly active preparation with one S. U. in 2.5 mg. In both series, the animals behaved equally.

As is evident from the table, about 60 per cent of the cats gave a satisfactory proportionality between the amount of gastrin administered and the acid produced.

In analyzing the material in table II, it was found that, in most of the 19 animals giving a less satisfactory response, the secretory mechanism had been exhausted through a previous large dose of gastrin. The same applies to many of the 24 cats, giving no proportionality at all: — thus, three of them had shortly before secreted 20, 42 and 69 ml of gastric juice respectively. Furthermore 6 of the cats in this group died within 2—3 hours during the experiment. As a consequence, there remained after a closer analysis of the whole material comprising 110 cats only 7 in which no explanation could be found as to why the secretory response had failed. In fact, there are cats which do not react even for 2—3 times larger doses of gastrin than those ordinarily used.

The exhaustion of the secretory mechanism was observed by UVNÄS. It was found to be particularly pronounced after the use

Table III.

The secretory response expressed in ml 1/10 n HCl after the first (I) and the sixth (VI) or seventh (VII) injection, the same amount of gastrin being injected both times.

Preparation nr. 55 60 mg/S. U.		Preparation nr. 62 60 mg/S. U.			
I	VI (VII)	I	VI (VII)	I	VI (VII)
6.0	3.0	5.5	1.5	9.0	6.5
6.0	6.0	6.4	2.5	3.2	4.0
7.4	7.0	5.0	6.0	4.5	4.5
7.5	4.0	8.5	5.0	11.5	5.75
6.5	0.5	5.2	4.0	7.0	2.5
11.5	4.5	7.4	5.0	12.5	11.0
10.0	6.0	8.0	4.8	7.5	7.0
12.0	6.0	7.0	2.0	5.0	7.0
7.5	6.5	12.7	8.0	11.0	5.0
6.5	1.0	8.0	8.0	9.0	5.0
9.0	9.0	14.0	3.0	17.1	5.0
5.2	3.0	6.5	4.0	14.0	4.25
8.6	3.0	9.8	9.8	21.0	6.5

of impure preparations of gastrin. It seems to follow in any cat after an excessive dose of gastrin.

In table III we have collected the material from about 40 experiments, each cat being given gastrin six or seven times with hourly intervals, the same dose of gastrin being given the first and the sixth (seventh) hour. In 9 of the cats, a very marked exhaustion was seen after six hours. In 13 of them, however,

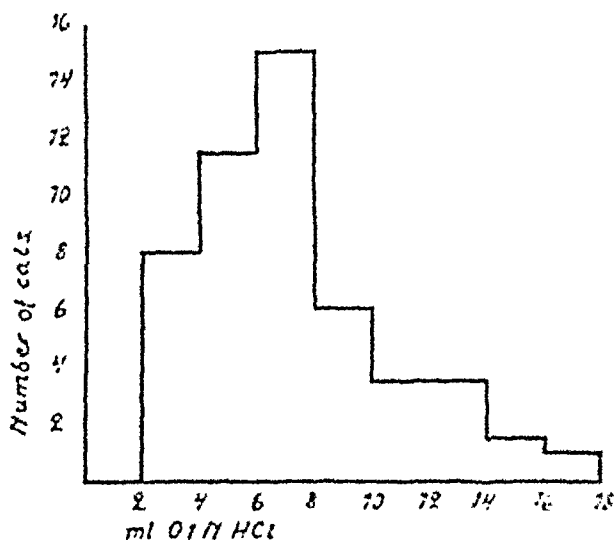


Fig. 1. The secretory response during the first hour to one and the same dose of gastrin in different cats.

the secretory response was as good after the sixth (seventh) injection as after the first. In most of the others, the effect was still good after six injections and could, as long as it did not fall below 3 ml be used in assaying unknown samples, due attention of course being paid to the weaker secretory response.

An attempt to use the cats even the following day after the operation gave less satisfactory results.

As in other biological standardizations, a reference substance, a *standard gastrin* is necessary here also. Not only do the individual cats give quite a different quantitative response to the injection of a definite amount of gastrin, as is seen in fig. 1, but they also vary in their response to repeated injections of gastrin. We therefore preferred to start every experiment with an amount of a standard gastrin, known to give 5–6 ml of 1/10 N HCl during the first hour. For the evaluation of the subsequent hourly

titrations, the same amount of gastrin was given in the sixth or the seventh injection.

Essential prerequisites for a successful assay of gastrin are consequently that:

1. The cats are in good health.
2. One hour is allowed to elapse after finishing the operation before the first dose of gastrin is given.
3. The initial dose of gastrin gives a convenient volume of gastric juice, 4—6 ml in one hour, allowing the dose to be doubled in a subsequent injection. Proportionality between the doses and the secretory responses must thereby be obtained. Otherwise smaller doses are tried.
4. Large doses of gastrin, causing exhaustion of the secretory mechanism, are to be avoided. The gastrin preparations to be assayed must be of a certain degree of purity, while an admixture of impurities causes an irregular and usually reduced secretion.
5. A standard preparation of gastrin is given in the first and the sixth (seventh) injection, the dose being adjusted so as to give preferably 4—6 ml of 1/10 N HCl during the first hour. The strength of the unknown is referred to this standard.
6. Not less than 3—4 (preferably 5) cats are used for the assay of one and the same sample of gastrin.

Summary.

Samples of gastrin were assayed on cats in urethane-chloralose anaesthesia in accordance with the technique of previous authors, KOMAROV and UVNÄS.

The secretory unit, S. U., *i. e.* the amount of gastrin producing 1 ml 1/10 N hydrochloric acid during one hour, was determined by injecting two different doses of the gastrin preparation, proportionality in the secretory response being demanded. Such proportionality appeared only within a narrow range, within which the first dose could not be more than doubled in a subsequent injection. In 60 per cent out of 110 cats good proportionality was obtained. In only 7 of the cats no explanation could be found why the secretory response failed. Some of the cats were in bad health and died early during the experiment. In most of the resistant cases, however, the lack of proportionality was due to

exhaustion of the secretory mechanism following a previous large dose of gastrin.

A standard gastrin was used as reference substance. An amount of this, known to give 4—6 ml 1/10 N HCl in one hour, was given in the first injection. In order to facilitate the evaluation of the response following the subsequent hourly injections of the unknown sample, and to control the secretory capacity of the animal during the experiment, the standard gastrin was given again in the sixth (or seventh) injection.

After a secretion larger than 10—12 ml during one hour an exhaustion of the secretory capacity was consistently seen during the next hours.

The variations in the individual response of different cats to one and the same dose of gastrin were demonstrated.

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An Electrolytical Method for the Determination of Cations.

By

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GIBBS (1880) was the first to suggest the determination of metals as amalgams by electrolysis, and SMITH (1903) carried out the first experiments of this kind.

HILDEBRAND (1907) for his experiments used a vessel with mercury in the bottom, into the mercury is dipped a cylindrical glass, and in this the solution to be analyzed is placed, and in the space outside for instance water. The mercury is the cathode and the anode is placed in the inner solution. The cations form amalgams with the mercury and these are destroyed in the outer chamber setting free the cations. Na, K, Ca and Ba could be transferred in this way, but not Mg.

STODDARD (1927) developed a micro method for the determination of sodium and potassium. The apparatus employed consists of two vessels separated by a glass wall with fine holes. Mercury is placed in the bottom of the two vessels, and connected with the anode and the cathode. The amalgams are formed in the cathode vessel and after electrolysis titrated with sulfuric acid. He found about 99 per cent of the added cations, but the method could only be used with ashed material.

ADAIR and KEYS (1934) altered the method so that the cations had to pass the pores of a collodium membrane. They determined the cations with the same exactitude and the method could also be used for serum as the membrane did not allow the proteins to permeate.

The membranes have to be tried as to their permeability not only after their making, but also later as they may alter by use. Later KEYS (1936) uses cellophane membranes as well and reports further determinations on salt solutions and serum and red blood corpuscles.

In most cases the analyses are carried out with 0.2 ml. and the results then are accurate to within 1 %.

NIELSEN (1940) also uses cellophane membrane, but HOLM-JENSEN (1943) finds that the blank values then increase to about 2 microequivalents, and are not constant. He modifies the method of ADAIR and KEYS. Analyzing 2 or more samples of 0.1 ml. of the same blood plasma, he finds that the difference will rarely exceed 0.3 microequivalents corresponding to 2 % of the contents of bases. Analyzing ashed and unashed samples most of the determinations will give the same agreement. The blanks contain less than 0.2 microequivalents.

HOLM-JENSEN also states a micro method using Hildebrand's principle, but the results were too low (about 3 to 6 per cent). Gentle stirring during the electrolyzation would diminish this error. For plasma determinations this method does not seem to be suitable. He finds that the collodium membranes can be used for 10 electrolyses. The method stated in this paper is based on the solubility of the amalgams in mercury as in Hildebrand's method, and the method to some degree resembles WIDMARK's rocking-extraction method (WIDMARK 1926, ØRSKOV 1928).

The Extraction Vessels.

The extraction vessels are made in the institute of "Duran" tubes with an inner diameter about 7 mm. The tube is intensely heated in the middle, drawn out and bent to become V shaped (See fig. 1), the legs are about 7 cm long. The tubes are cleaned mechanically, in concentrated HNO_3 , distilled and redistilled water, and dried with compressed air.

The Rocking Apparatus.

The rocking apparatus is seen in fig. 2. It performs about 25 rocking movements (of 30°) per minute. When the tubes are not rocked the extraction is not so perfect that it can be used for a quantitative analysis. Still it is astonishing that the electrolysis

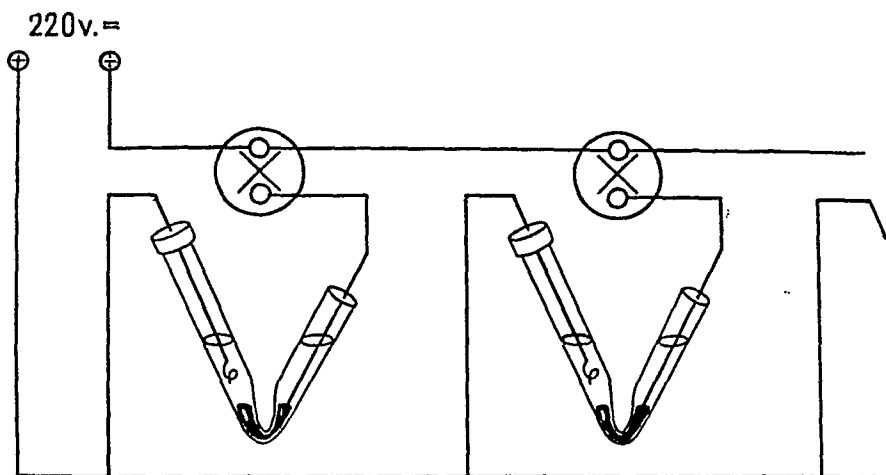


Fig. 1.

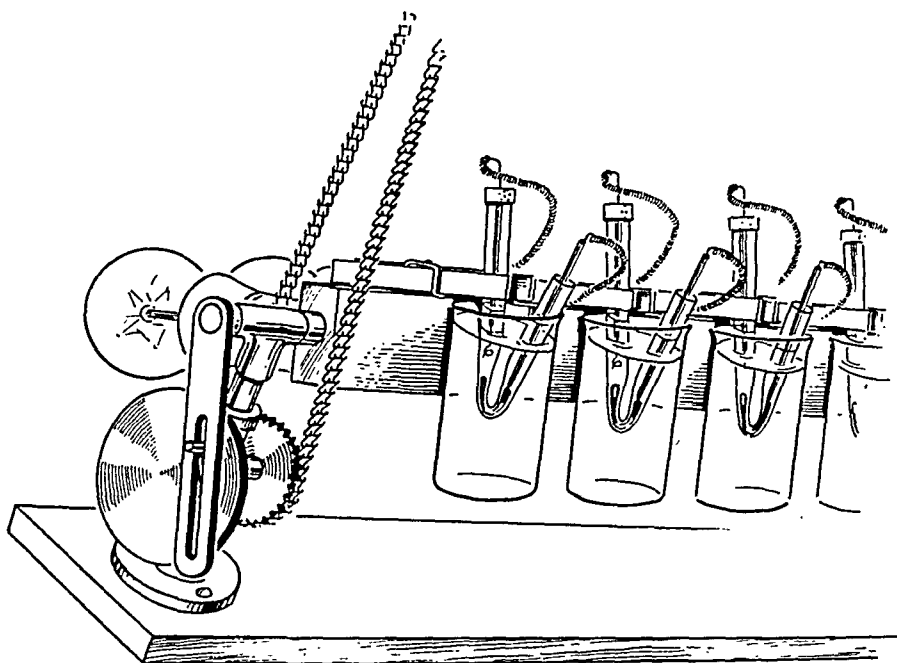


Fig. 2.

(the heat production and the hydrogen formation in the anode tube) results in a relatively high degree of extraction caused by lively movements of the mercury. The tube sits in a clothes-peg, which is fastened to the axis. The electro-motive force for the electrolysis is 220 volt but much less may be used, the procedure then being slower, and as resistance a 15 watt bulb is connected.

with each tube (see fig. 1). For cooling the lower part of the tube is placed in a beaker with water, the beaker being fastened to the axis with a rubber string. 12 analyses can be carried out at a time.

Procedure.

First 1 ml. of mercury is placed in the V tube thus separating it in two. Into one of the branches (the cathode tube) is measured 1 ml of 0.02 N H_2SO_4 by means of an automatic syringe (KROGH 1935), into the other branch (the anode tube) the same amount of the fluid to be analyzed. The electrodes, which are covered with thin "Duran" tubes melted round a platinum wire piece in the end, are now placed in the tubes, the cathode so that the platinum is covered by the mercury, and the anode so that the platinum wire is in the upper part of the solution. When the extraction is finished the electrodes are removed, the fluid adherent to the cathode is flushed into the cathode tube with a few drops of water, a drop of methylred is added to the cathode tube, and the free acid here is titrated with the WIDMARK-ØRSKOV burette (1928) — the point of the burette dipping into the solution, using 0.05 N NaOH. Throughout the titration a fine current of CO_2 -free air bubbles from a fine tube reaching the mercury mixes the solution. As a small part of the cations is set free between glass and mercury, it is of importance before titration several times to let the tube with the bubbling air slide between the wall and the mercury. The cations will then be transferred to the liquid above.

If the solution to be examined foams during the electrolysis, the anode tube is used 1.5 cm longer (as seen from the figures), and 0.1 ml. of octyl alcohol is added.

It has been tried to reduce the volume of mercury, and in a large number of experiments only 0.2 ml. was used. The transfer of the cations is then much faster (15 minutes for total extraction of serum, and the values found are the same as with 1 ml. mercury and 1 hours extraction), but in many cases water will then pass from the anode tube to the cathode tube, especially when the solution in the anode tube is diluted acid. The transfer of water may also happen with 1 ml. of mercury.

If a solution of for instance NaCl is electrolyzed most of the sodium is transferred in the first few minutes; the procedure can be followed as the sodium amalgam on the surface of the mercury in the cathode tube is split and hydrogen set free.

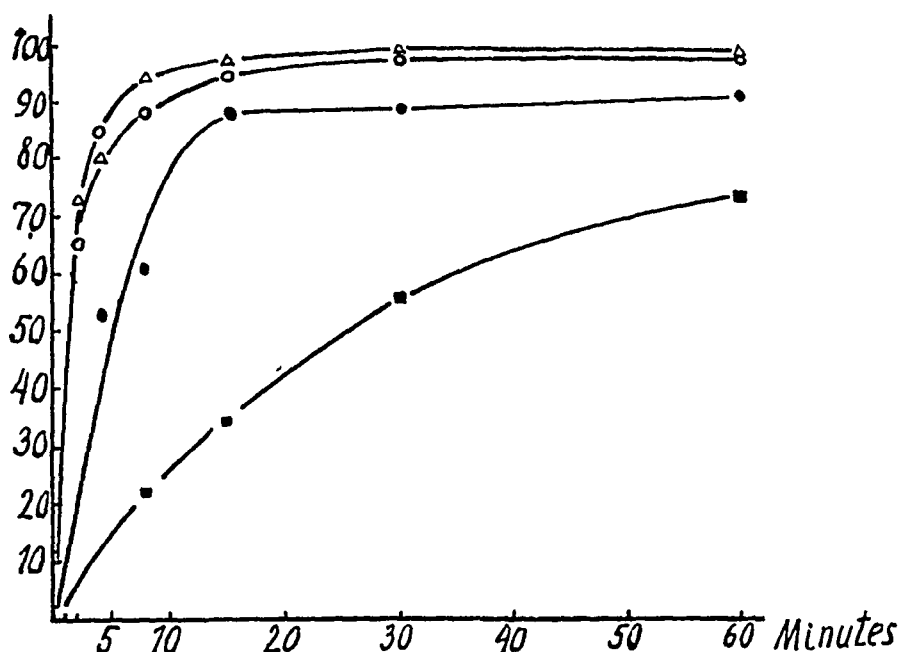


Fig. 3. Extraction-rates of sodium = \triangle — \triangle — \triangle , potassium = \circ — \circ — \circ , calcium = \bullet — \bullet — \bullet and ammonium = \blacksquare — \blacksquare — \blacksquare . Ordinate per cent recovered.

The mercury employed is distilled, and after use purified in the double volume diluted nitric acid, a current of air being pressed through the mercury for 1 hour, and then in the same amount of distilled water, which is changed 4 times; finally the mercury is filtered through a fine hole in a paper filter.

The Rate of Transfer of the Cations.

One ml. 0.00612 n solution of the chlorides of the different cations is placed in the anode tube by means of an automatic syringe. In the controls the same amount of hydrochloric acid of the same normality is measured (the amount of acid transferred to the cathode tube is insignificant), and four analyses of each kind are carried out.

The tubes are rocked for varying times and titrated. From the difference in the titrating values the amount extracted can be calculated. The results are seen in fig. 3.

As to sodium and potassium 75 per cent are extracted after $2\frac{1}{2}$ minutes. After 1 hour 100 per cent of sodium are recovered, of potassium between 98 and 99 per cent. 50 per cent of calcium

are extracted after about 5 minutes. After one hour somewhat more than 90 per cent are extracted.

The reason for this imperfect extraction can easily be seen. Very soon after the rocking has begun a greyish layer is formed where the mercury and the glass are meeting, and this layer only partly disappears during rocking. It seems as if the calcium amalgam is easily precipitated on the glass wall. This precipitate does not hinder the extraction of other cations. Magnesium is not extracted at all. The magnesium amalgam stays as a darkish grey layer on the top of the mercury in the anode tube. The ferric ion is not extracted at all. Ammonium is only slowly extracted, 50 per cent after 25 minutes and about 75 per cent after one hour.

Apart from these cations some substances, which might be extracted have been tried. Creatinine is not extracted, but guanidine is, which can be attributed to its stronger basic properties. Histidine and morphine are not extracted. Sulphate and phosphate do not hinder the extraction.

Determination of the Cations of Serum and Red Blood Corpuscles.

Determinations have been made on the blood from a normal person and were partly carried out on serum and blood corpuscles, which had been ashed, and partly directly on the serum and the erythrocytes. As to the methods of ashing and procuring of red blood corpuscles, see former publications (V. HENRIQUES and S. L. ØRSKOV, 1936 and S. L. ØRSKOV 1946 1 and 2).

Through a large number of experiments it was shown, that in order to get a perfect extraction it is of importance that the proteins are precipitated during the electrolysis. This is achieved for serum by adding alcohol and a little acid, for erythrocytes by adding alcohol. Serum is diluted 1 to 20 with a solution consisting of 2 parts of 0.1 N HCl and 18 parts of 96 per cent alcohol. The solution must not be too acid, if it is for instance 0.1 N HCl no extraction at all takes place, perhaps because the amalgams are immediately broken down after being formed. 1 ml. is taken for each analysis with an automatic syringe. One volume of the red blood corpuscles is first haemolyzed in 4 volumes of H_2O , 0.25 ml. of the solution is transferred to the anode tube with a syringe, and there 0.75 ml. of 96 per cent alcohol is added. The alcohol also prevents the foaming.

For the controls the diluting fluids are used. The blanks contain about 0.05 microequivalents, by the direct determinations essentially less than this. The rocking time is 1 hour.

In table 1 the results from the same blood sample are given. After defibrination serum and cells were separated and kept at 3°.

Table 1.

<i>Ashed</i> ¹		<i>Serum.</i>	<i>Direct Determination</i> ¹	
Cations in m.eq.	Average	Cations in m.eq.	Average	
147 ± 1.1	148	148 ± 0.7	150	
150 ± 0.8		149 ± 0.7		
146 ± 1.2		151 ± 0.9		
150 ± 0.8		152 ± 0.6		
148 ± 0.7				
<i>Ashed</i> ¹		<i>Erythrocytes.</i>	<i>Direct Determination</i> ¹	
Cations in m.eq.	Average	Cations in m.eq.	Average	
102 ± 0.8	101	107 ± 1.0	107	
102 ± 1.0		105 ± 0.4		
102 ± 1.0		108 ± 0.6		
99.3 ± 0.4				

It is seen from table 1 that the accuracy is higher by the direct determination than on ashed material. The experiments are spread over a week and during this period the values found by direct determination are increasing with the exception of the first value of the erythrocytes.

Perhaps bases are formed during this time.

The averages differ more than is consistent with the accuracy of single determinations according to table 2. This is supposed to be caused by the ashing and by the preparation of the solutions.

Table 2.

Serum cations in m.eq. by direct determination.		Erythrocyte cations in m.eq. by direct determination.	
Values of single analysis from one experiment.	Average	Values of single analysis from one experiment.	Average
153	152 ± 0.6	108	108 ± 0.6
153		111	
154		108	
151		110	
150		109	
150		107	
151		107	
153		108	

The accuracy of single analysis is ± 1.6.

¹ The values refer to the same blood sample. 5 portions of the serum and 4 of the erythrocytes have been ashed, and after dilution 4 single analyses on each have been made. The averages of these are given above. The values found by direct determinations are the averages of 6—8 analyses.

In the case of serum the values found by direct determination and after ashing are almost the same.

As to the erythrocytes the values by direct determination are about 6 m. eq. higher than found after ashing. There may be two reasons for the higher values found by direct determination 1) there are organic bases in the erythrocytes, or such bases are formed during the electrolysis, 2) in the not insignificant amount of insoluble ash, sodium and potassium, which are determined by the direct determination, are bound, or potassium is lost by the ashing. (This will not amount to 6 m. eq.)

The Applicability of the Method.

For the determinations of the total amount of the cations in serum the method can be used in the clinics, as it is exact and can be used for routine analysis.

In biological experiments on blood it gives the total amount of the cations without ashing, which is always a delicate affair. Magnesium is not determined by the method, there is no calcium in the erythrocytes, and the calcium content of serum is in most cases constant, so that a determination of either sodium or potassium in the extract will give the contents of the other metal.

The method may be used, perhaps with a modification, for purifying a solution of cations.

Some experiments have been carried out to see if it can be used for the determination of the cations in muscles. This, of course, can be done after ashing, but a direct determination method has not been developed still.

If frog muscles are frozen in liquid air, ground and suspended in alcohol with acid, it is found that a proportionately big part of the cations is still bound to the muscle proteins after 24 hours. An extraction with trichloroacetic acid can not be used, as even traces of this acid prevent any extraction. Further experiments are needed to clear the problems. It must always be kept in mind that the electrolysis or the precipitation of the proteins may give rise to the formation of basic substances, which were not preformed in the organs.

Summary.

The method is based on the solubility in mercury of the amalgams of the cations formed by electrolysis. The amalgams are extracted by rocking movements (WIDMARK) and titrated.

The extraction velocity for some cations is determined.

Sodium (100 per cent) and potassium (98—99 per cent) are recovered after one hours rocking. After the same time 90 per cent of calcium are extracted, some of the calcium amalgam being precipitated on the glass wall.

Ammonium and guanidine are also extracted, but only slowly, magnesium and ferric ion can not be extracted.

Direct determinations can be made on serum and erythrocytes, it is important that the proteins are precipitated during the electrolysis. There is good agreement with the values found after ashing of serum; direct determination on the erythrocytes give a somewhat higher value than after ashing.

The analyses are carried out with 0.05 ml. serum.

The applicability of the method is discussed.

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Non Absorption of Finely Emulsified Paraffinum Liquidum in the Intestine of the Rat.

By

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FRAZER's "Partition Hypothesis" of fat absorption (1946) can be resumed as follows:

The fats normally present in the food are absorbed in two different ways. When fat is introduced into the intestine a finely dispersed emulsion is produced. From this emulsion fat is absorbed partly as very small unhydrolysed globules, and partly as hydrolysis products, *viz.* glycerol and fatty acids. These three products pass through the epithelium of the intestine. Emerging from the cells the fat absorbed in the form of globules passes directly to the lymphatic ducts where it is visible as a white emulsion; from the thoracic duct this part of the fat enters the systemic blood. The fatty acids on the other hand pass, without resynthesis to triglyceride, to the central vein of the villus, *i. e.* to the portal system. FRAZER thinks that the greater part of the fat is absorbed in the first way, that is in the form of finely dispersed unhydrolysed globules.

This theory is based on experiments in which three different methods have been used: histological and chymographic investigations of the absorption of triglyceride (FRAZER 1943) and absorption experiments with finely emulsified paraffin (FRAZER 1942, 1944). In this paper only experiments with paraffin absorption will be dealt with.

The main point of the theory is, that fat *can* be and that normally the major part of it *is* absorbed as unhydrolysed globules. Consequently it would be a very strong support of the "Partition

Hypothesis" if it could be shown that unhydrolysable hydrocarbons could be absorbed from a suitably fine emulsion.

In his experiments FRAZER found that as much as 36 % was absorbed when a specially prepared paraffin emulsion was injected into the duodenum of rats. In similar experiments with oil the absorption amounted to 33 %.

In addition to these quantitative experiments FRAZER states (1942, 1946 a and b) that absorption of paraffin can be demonstrated by means of histological examination of the epithelium of the intestine. As regards chylomicrographic investigations a preliminary report (1942) tells that following the administration of paraffin a rise in the number of chylomicra in the blood is observed, just as is the case when olive oil is used. In the final report these results are not mentioned, and in a lecture (1946 b), which has been published after our experiments were finished, it is now stated: "... the paraffin has only been satisfactorily transported from the lumen through the outer border into the intestinal cell. Whether it is possible to carry the paraffin further along the absorption pathway has yet to be established."

The results obtained by FRAZER with paraffin emulsions completely disagree with those of other investigators, who seem always to have had negative results in that kind of experiments (HENRIQUES and HANSEN 1900, BLOOR 1913, MELLANBY 1927).

FRAZER is of the opinion that the reason why he has found absorption of paraffin is, that his emulsions contain smaller particles and are more stable than those employed by others.

With the exception of FRAZER's experiments the literature contains no reports indicating a significant absorption of unhydrolysed fat or paraffin. MELLANBY (1927) found that triglyceride was absorbed from the intestine of the cat, even after ligation of the pancreatic duct. In his opinion the intestine of the cat is almost free from intestinal lipase, and he therefore concluded that the fat was absorbed without preliminary hydrolysis. It has, however, later been shown that the intestine of the cat contains sufficient amounts of lipase to account for the absorption observed (VERZAR 1931). It has been shown, with ordinary biochemical methods (CHANNON and COLLISON 1929, CHANNON and DEVINE 1934, MAHDI and CHANNON 1933), as well as with isotope technique (STETTEN 1943), that hydrocarbons *can* be absorbed from the intestine. These experiments, showing absorption of very small amounts of hydrocarbon which were given in the food over a period of several days, are of quite another character than those of FRAZER, and they are not suited to solve the problem whether

or not unhydrolysed fat is normally absorbed in great quantities from the intestine.

In the following the results will be given of experiments in which it was attempted to confirm the results of FRAZER's paraffin experiments.

Experimental Technique.

Paraffin emulsions were prepared *a. m.* FRAZER (1944) by dissolving 4 % oleic acid and equimolar amounts of cholesterol in hot paraffinum liquidum, and pouring the hot mixture into an equal volume of a 1 % solution of sodium carbonate. This procedure leads to the spontaneous formation of an emulsion, which is then passed through an emulgator. — We first employed an emulgator built according to the diagram given by FRAZER and WALSH (1933); with this apparatus we were, however, unable to produce emulsions with an average particle diameter as small as it should be after FRAZER's descriptions. More satisfactory results were obtained by the use of an industrial machine for dairy products. With this machine, which works at about the same pressure as FRAZER's emulgator (200 atm.), but is fitted with a more complicated device for subdivision of the globules, emulsions were formed with particles averaging 0.3—0.5 μ . To obtain such an emulsion we had, however, to use paraffinum liquidum tenue; with the more viscous paraffinum liquidum emulsions were produced which were less homogeneous and had a larger average diameter of the globules than those described by FRAZER. In the absorption experiments we therefore only used emulsions prepared with paraffinum liquidum tenue. — Our best emulsions were obtained when the crude preparation was circulated in the machine for about 6 min.; microscopical and viscosimetric examinations showed, that after this period, which corresponds to about 50 passages, no further improvement could be demonstrated.

Having seen but negative results with emulsions prepared as just described, new experiments were set up, in which we used an emulsion which according to a personal communication from Professor FRAZER should be more advantageous than the one used by him in his own experiments (1944). This new emulsion was prepared as follows: 2 g of cholesterol is dissolved in 200 ml of paraffin and the mixture heated to 70°. 5 ml of oleic acid is then added, and the oil phase is poured into 200 ml of a solution containing 1.5 g anhydrous sodium carbonate in distilled water. This mixture is shaken vigorously and then passed through the emulsifying machine at maximum pressures. After preparation the emulsion is placed in a separating funnel for 72 hours. The lower part of the emulsion is used, and if the preparation is successful very little creaming should occur. In our case preparation according to this prescription resulted in emulsions which were no more and possibly somewhat less satisfactory than those prepared by the method originally given by FRAZER.

For the actual absorption experiments rats were used, which had been fasted for 24 hours. In light ether anesthesia the emulsion was injected into the duodenum; the injection was made through double ligatures at the pyloric ring, and another ligature was placed at the iliocolic junction. After the operation, which lasted only 3—5 min., the animals were left in separate cages for 6 hours when they were killed and the intestines removed. From an irrigator an alcohol-ether mixture was run through the gut thus breaking the emulsion instantaneously and transferring the total contents of the gut to test tubes. After evaporation of the ether-alcohol on a water bath, the amount of ether soluble substance in the residue was determined by weighing. For comparison determinations were made on the original emulsion, and in order to test the completeness of the emptying of the intestines by the method employed, control experiments were carried out in which emulsion was injected into freshly removed guts, rinsed out and analysed as described above.

In some experiments the contents of the intestines were examined microscopically after the absorption period, and it was found that the emulsion contained in the upper, more acid part of the intestine had been broken down considerably. In the lower part of the intestine some of the emulsion had deteriorated a little, but larger amounts were found still to be in the original form of a very finely dispersed emulsion. In consequence of these findings experiments were carried out in which only the lower $\frac{2}{3}$ — $\frac{3}{4}$ of the intestine were used; for these experiments we used emulsions prepared according to FRAZER's original prescription.

Experimental Results.

The results of our experiments will be found in Tables 1—3, which are largely selfexplanatory. It is seen that in no case has

Table 1.

Ether soluble material found in	
Control animals	Absorption animals
g. 2.325	g. 2.361
2.347	2.339
	2.354
av. 2.336	2.341
	2.444
	2.319
	2.392
	2.355
	av. 2.363

Total small intestine. — About 5.5 ml of emulsion a. m. FRAZER. For the measuring off of the emulsion — here as in the following experiments — a syringe with a movable stopper which could be fixed at any level was used. Weighing of the filled syringe after successive fillings revealed differences of only a few mg.

Table 2.

Ether soluble material found in		
Emulsion	Control animals	Absorption animals
g. 1.036	g. 1.121	g. 1.172
1.091	1.041	1.135
1.054	1.075	1.179
1.115	1.196	1.130
1.029		1.100
av. 1.065	av. 1.108	1.135
		1.088
		1.115
		1.138
		1.118
		1.135
		av. 1.131

Total small intestine. — About 3 ml of emulsion prepared after special prescription (see text).

absorption of the injected paraffin taken place. Considering the accuracy of the determinations, as expressed by the standard deviation from the mean, demonstration of absorption percentages as low as 8—10 % should be possible. Percentages of this magnitude were in FRAZER's experiments obtained with emulsions much coarser than those employed in our experiments.

Table 3.

Ether soluble material found in		
Emulsion	Control animals	Absorption animals
g. 0.816	g. 0.835	g. 0.881
0.826	0.840	0.888
0.831	0.866	0.904
0.784	0.860	0.856
0.811	0.796	0.883
	0.885	0.875
av. 0.814	av. 0.850	av. 0.881

Distal $\frac{2}{3}$ — $\frac{3}{4}$ of small intestine. — About 2 ml of emulsion a. m. FRAZER.

A "partial" absorption, *i. e.* a passage of paraffin globules into the intestinal cells in accordance with FRAZER's latest interpretation of his own results, can of course also be excluded in our experiments.

In all cases the blank values were found to be a little lower than the values determined from the intestines. This is probably due to the fact that small amounts of ether soluble material is contained in the intestine of 24 hours fasted rats. On analysis of the contents of guts into which no paraffin had been injected, ether soluble material was found in amounts varying between 50 and 100 mg.

Summary.

On injection into rat duodenum of emulsions of paraffinum liquidum tenue, prepared *a. m.* FRAZER, and with an average globular diameter of 0.3—0.5 μ , no absorption was found over a period of 6 hours.

It has thus been impossible to confirm this important support of the "Partition Hypothesis".

Our thanks are due to H. H. USSING, Ph. D., of the Laboratory of Zoophysiology, University of Copenhagen, for valuable advice during the performance of these investigations.

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The Concentration of Blood Lactic Acid in Man during Muscular Work in Relation to the Partial Pressure of Oxygen of the Inspired Air.

By

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If a subject performs muscular work of a sufficiently high intensity he will get an increased concentration of lactic acid in his working muscles and an escape of this lactic acid (as lactate) into his circulating blood. If the work is of constant intensity and is continued for a sufficiently long time, the blood lactic acid concentration will reach a steady state after a temporary rise above this level at the beginning of the work: the rate of lactic acid escaping from the active muscles will then be in equilibrium with the rate of lactic acid assimilation and dissimilation in the tissues (ESKILDSSEN 1945). The rise in concentration of the blood lactic acid from the rest value to the steady state is quantitatively dependent on several factors. One factor is the intensity of the work: the higher the intensity the higher the rise of lactic acid. Another factor is the subject's state of training: the better the state of training the smaller the rise of lactic acid at a given intensity of work (OWLES 1930, ROBINSON and HARMON 1941).

It has been shown experimentally that an insufficient supply of oxygen to working or resting muscles causes an increased formation of lactic acid (FLETCHER and HOPKINS 1906, PIERY et al. 1939). The rise in blood lactic acid during muscular work can thus be explained by an increased anaerobic carbohydrate decompo-

sition due to anoxia in the active muscles, and factors that tend to increase the anoxia of the muscles will also cause an increased lactic acid formation. Such factors are anoxic, anemic, stagnant and histotoxic anoxia.

The importance of anoxic anoxia for the lactic acid formation in an organism has been studied by several investigators. The concentration of blood lactic acid in subjects staying for a certain time on mountains at different altitudes was studied by LAQUER (1922) and by BAICENKO and KRESTOWNIKOFF (1933) among others. These early investigations, however, did not give clear information about the lactic acid level during rest and work at high altitudes.

DILL, EDWARDS et al. (1931) made experiments on subjects staying at an altitude of 3,050 m for 6—8 weeks. The subjects worked for 20 min. on a bicycle ergometer, and the oxygen consumption and the blood lactic acid level were determined at the end of the work. At that altitude the subjects had a diminished capacity for work, and for a given oxygen consumption (*i. e.* for a given intensity of work) they showed a higher rise of blood lactic acid than at sea level. In one of the subjects, however, a continuous change during the 8 weeks' stay occurred: the extra rise of lactic acid mentioned became smaller and eventually disappeared, so that at the end of the stay the subject showed the same blood lactic acid rise for a given intensity of work as at sea level. During this stay the subjects' hemoglobin concentrations changed only very slightly, and the concentrations of plasma bicarbonate were diminished by an average of 2 m ekv/l.

EDWARDS (1936) continued these investigations of the effect of acclimatisation. Experiments were made on subjects staying at an altitude of 2,800 m for 6 weeks, and later at 3,660 m, 4,700 m, and 5,340 m. Work was performed on a bicycle ergometer for 10 min. At the end of the work the blood lactic acid was determined. At the beginning of the stay at 2,800 m 3 out of 7 subjects had a tendency to produce extra lactic acid at a given grade of work, as compared with the values at sea level, but after 6 weeks at 2,800 m and at all the higher altitudes given above all the subjects showed the same lactic acid rise as at sea level for a given grade of work. With increasing altitudes the maximal capacity for work was constantly reduced and consequently the lactic acid rise after maximal work intensity was also diminished. The result of EDWARD'S investigation (1936) is partly contradictory to that of

earlier investigations (BAICENKO and KRESTOWNIKOFF 1933, DILL, EDWARDS et al. 1931) but EDWARDS allowed his subjects to acclimatise for a longer time than earlier investigators had done. EDWARD'S conclusion is that "it seems highly significant that for a given grade of work anoxemia does not produce a greater than normal increase in lactic acid", with the qualification that this statement is valid generally only after acclimatisation.

It is the aim of the present investigation to study the concentration of lactic acid in the blood of subjects performing work for a sufficiently long time to ensure a steady state of lactic acid, with varying grades of work and varying oxygen partial pressures of the inspired air.

Methods.

The experiments were performed on two students, physically fit and in good training, subjects P. and Th. The subjects previously had done similar work on the bicycle ergometer, and to ensure as constant a state of training as possible experiments were performed 5 days a week. The experiments were made in the morning and the subjects came to the laboratory directly after getting up and after a standardised morning meal, consisting of milk and bread-and-butter. Before the beginning of the experiments the subjects rested on a bed for at least half an hour. The work was performed on a Krogh bicycle ergometer with varying loads and a constant pedalling frequency, 60 per minute. An experiment of this type generally lasted 45—50 min. Stress was put on the fact that the work should be performed with as few unnecessary movements of the arms and the body as possible. In this respect subject P. was ideal. The oxygen consumption during work was measured by collecting samples of expired air in Douglas bags taking 75—100 l. Samples were taken generally after 15, 30 and 45 min. of work, sometimes after 20 and 40 min. When a sample was taken two rubber bags were filled. During the experiments with an altered partial pressure of oxygen in the inspired air, the subjects breathed an oxygen-nitrogen mixture through the mouthpiece from a rubber bag which was filled from a cylinder. The subjects breathed the mixture for some minutes before starting the work. Generally the subjects could easily manage the mouthpiece without leakage, but during the experiments with a very low oxygen partial pressure they were mentally confused and a most careful control had to be exerted. Leakage during inspiration from mixtures with a low oxygen percentage gives too high an oxygen percentage for the samples of expired air, too low an oxygen difference and too low an oxygen consumption value. Leakage during expiration gives too low ventilation values and also too low an oxygen consumption value. The following mixtures of oxygen and nitrogen were used:

Oxygen per- centage %	Oxygen partial pressure (at 760 mm Hg) mm Hg	Corresponding altitude above sea level metres
10.5	80	5500
12	91	4450
14	106	3150
16	122	2150
17.5	133	1400
47	357	—

Analyses of samples of the oxygen mixtures when the cylinders were full and when they were emptied showed a variation of less than 0.02 % O_2 , *i. e.* a variation within the limits of the analytical error. The analyses of the oxygen and the carbon dioxide percentages of the samples of expired and inspired gas mixtures were made with Haldane's gas analysis apparatus as modified by Krogh. The analytical error of this method is less than ± 0.02 % O_2 and ± 0.02 % CO_2 . If a sample contains more than 45 % $O_2 + CO_2$ it must be diluted with N_2 , which diminishes the analytical accuracy.

Blood samples for the determination of the blood lactic acid were taken from a fingertip which had been arterialised by the hand being kept in hot water (46° C.) for 5 min. By this procedure the capillary blood becomes almost arterial. A small stab was made and the blood was collected on a small glass vessel with a few stains of heparin. 0.5 ml of blood was immediately pipetted off for determination. This was made according to the method of EDWARDS (1938), a slight modification of the method of FRIEDEMANN, COTONIO and SHAFFER (1927). A recent survey of this method has been given by ESKILDSEN (1945). For the amounts of lactic acid in question, more than 10 mg% in whole blood, the analytical error is less than ± 10 %. Blood samples were taken during rest just before the beginning of the work and during work with intervals of 5—10 min. At the end of the experiment, when the lactic acid content of the blood was supposed to have reached a steady state, two successive samples were taken. Double analyses of each sample were generally made.

Results.

The results are shown in tables I and II. The oxygen consumption during constant work, as measured after 15, 30 and 45 min. of work, did not vary more than ± 3 %. The values in column 5 are the oxygen consumption values at the end of the work. In column 4 the corresponding ventilations are shown. The oxygen consumption for a given grade of work did not vary more than ± 6 % in the case of subject P., in spite of the changes of the inspiratory oxygen pressure. The experiment of June 7th is an exception, though, in this respect. It might be explained by a

leakage between the lips and the mouthpiece during expiration, as this would give too low oxygen consumption values, but such a leakage was not observed. Subject Th. showed, however, greater variations in oxygen consumption values for a given grade of work, amounting to $\pm 10\%$. The explanation probably is the one mentioned above, leakage between the lips and the mouthpiece. Subject Th. was often very restless on the bicycle and therefore difficult to supervise. He also had a tendency to perform unnecessary movements, which would augment the difference in oxygen consumption values. Theoretically one would expect to find raised oxygen consumption values for a given grade of work in experiments with low inspiratory oxygen pressures, as in these the ventilation is correspondingly increased and consequently the oxygen consumption of the respiratory muscles is augmented. A raised minute volume of the heart, compensating the reduced oxygen saturation of the hemoglobin, would also give a relatively increased oxygen consumption by the heart. Such a difference has not been found, and would have required a greater accuracy in the measuring of the oxygen consumption. In the experiments of May 7th and 27th, subject P. working 900 kgm/min., the ventilation was increased, compensating the reduced oxygen pressure, from 41.8 l/min. (estimated oxygen consumption of the respiratory muscles: 75 ml/min) to 65.3 l/min (175 ml/min). The expected increase in oxygen consumption would be about 0.1 l/min, less than 4% of the total (NIELSEN 1936).

In column 6 the blood lactic acid concentrations during rest are given. They vary between 8 and 18 mg%, indicating that absolute resting conditions were not always attained before the experiments. In column 7 the steady state lactic acid values are given, both the lactic acid concentrations when a steady state is attained and the concentrations when the work is completed. In column 8 the points of time when the two steady state samples in column 7 were taken, are given in minutes from the start of the work. If in an experiment no steady state was reached, the final values are shown in brackets in columns 7 and 8. In all experiments except four a steady state of lactic acid level was reached. The time from the start of the work to the commencement of the steady state period varied between 0 and 45 min. with no special relation to the grade of work or to the inspiratory oxygen partial pressure.

It seems quite clear from the results that a reduced inspiratory

Table I.
Subject P.

Date	Grade of work kgm/min.	Oxygen % insp air	Ventilation l/min.	O ₂ consump- tion l/min.	Blood lactic acid, mg %		Steady state after start in min.
					rest	steady state	
May 16th	rest	20.9	6.4	0.295	10.2	—	—
May 14th	360	20.9	23.3	1.17	15.9	15.9—16.5	0—42
May 28th	360	10.3	23.5	1.05	—	16.1—17.0	12—60
May 13th	720	20.9	34.1	1.83	16.1	16.1—14.2	0—40
May 15th	720	12.0	42.6	1.82	17.3	17.3—14.8	0—42
May 21st	720	10.3	43.7	1.64	18.2	(—40.9)	(0—50)
May 6th	900	20.9	42.7	2.22	14.3	14.3—14.9	0—46
May 7th	900	14.6	41.8	2.19	12.6	18.7—21.8	25—33
May 27th	900	10.6	65.3	2.24	13.1	68.5—70.8	30—50
June 6th	1080	47.5	44.6	2.42	6.8	6.8— 8.5	0—50
May 2nd	1080	20.9	49.6	2.64	10.9	21.8—20.7	35—46
May 20th	1080	20.9	48.5	2.53	15.3	22.7—22.7	45—50
May 29th	1080	17.6	51.0	2.42	10.7	18.2—19.9	10—52
May 3rd	1080	14.5	57.4	2.57	11.9	41.7—39.6	35—45
May 8th	1260	20.9	64.2	3.09	13.8	43.0—42.5	45—52
June 3rd	1260	20.9	54.5	2.88	14.8	29.0—30.1	20—48
May 31st	1260	17.2	62.1	2.81	8.0	36.9—34.1	40—53
May 10th	1260	13.4	70.1	2.77	9.8	57.9—55.6	15—52
June 5th	1260	13.9	73.4	2.94	9.1	42.0—40.4	47—52
June 7th	1440	47.5	57.3	(2.79)	8.5	17.6—17.1	20—52
May 16th	1440	20.9	75.5	3.98	10.2	46.6—45.1	40—55

Table II.
Subject Th.

Date	Grade of work kgm/min.	O ₂ -% of insp air	Ventilation l/min.	O ₂ consump- tion l/min.	Blood lactic acid, mg %		Steady state after start in min.
					rest	steady state	
May 20th	rest	20.9	5.35	0.273	10.2	—	—
May 14th	360	20.9	23.5	1.24	15.3	11.4—11.4	15—41
May 13th	720	20.9	34.5	1.74	17.2	14.9—12.6	10—39
May 15th	720	12.4	42.3	1.82	15.3	15.3—16.5	10—40
May 8th	900	20.9	42.3	2.27	12.6	16.1—16.1	5—49
May 21st	900	16.0	43.6	2.17	13.6	18.2—20.5	30—50
May 10th	900	12.1	50.7	2.17	10.9	36.7—37.9	25—43
June 14th	1080	47.6	42.0	2.14	14.2	18.2—18.2	10—50
May 6th	1080	20.9	57.6	2.75	11.5	25.2—31.5	30—62
May 20th	1080	20.9	50.3	2.61	10.2	27.3—25.0	30—46
May 23rd	1080	16.2	51.6	2.47	13.1	27.8—29.0	40—48
June 6th	1080	13.7	56.4	2.54	10.8	(—47.8)	(—51)
May 31st	1080	11.8	61.2	2.15	10.2	(—51.7)	(—20)
May 16th	1260	20.9	69.4	3.31	15.4	(—40.9)	(—52)
June 4th	1260	20.9	57.7	3.12	10.4	25.6—27.3	30—52
June 3rd	1260	16.5	56.0	2.86	11.4	34.1—32.4	20—48
June 13th	1440	47.4	53.4	3.12	13.5	25.8—27.3	20—49
June 12th	1440	20.9	58.1	3.11	10.8	39.2—39.0	10—47

oxygen partial pressure produces a greater than normal increase in blood lactic acid for a given grade of work, if the oxygen pressure is reduced suddenly as in these experiments.

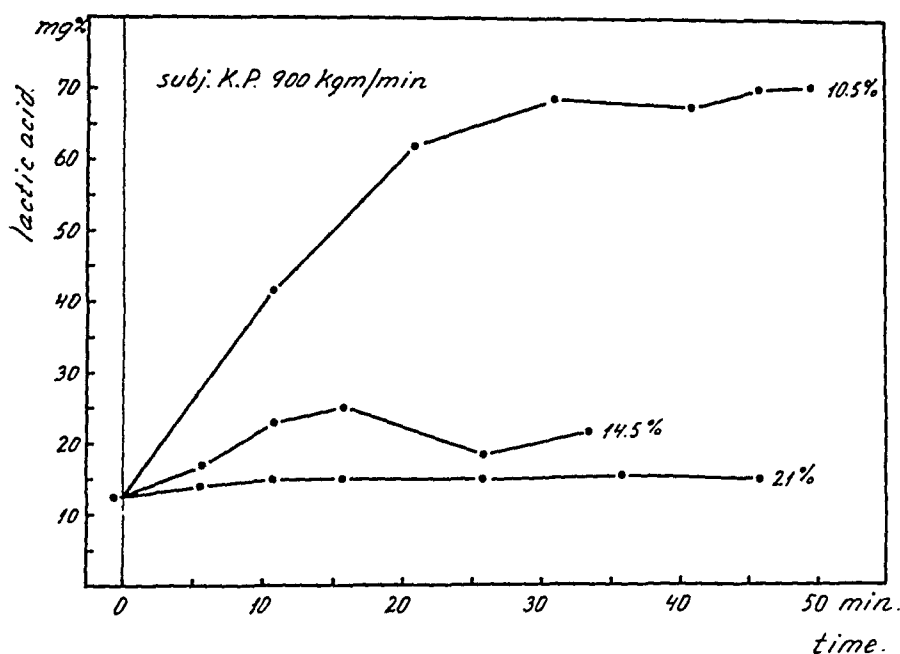


Fig. 1. Blood lactic acid of subject P. working 900 kgm/min. with varying oxygen percentages in inspired air.

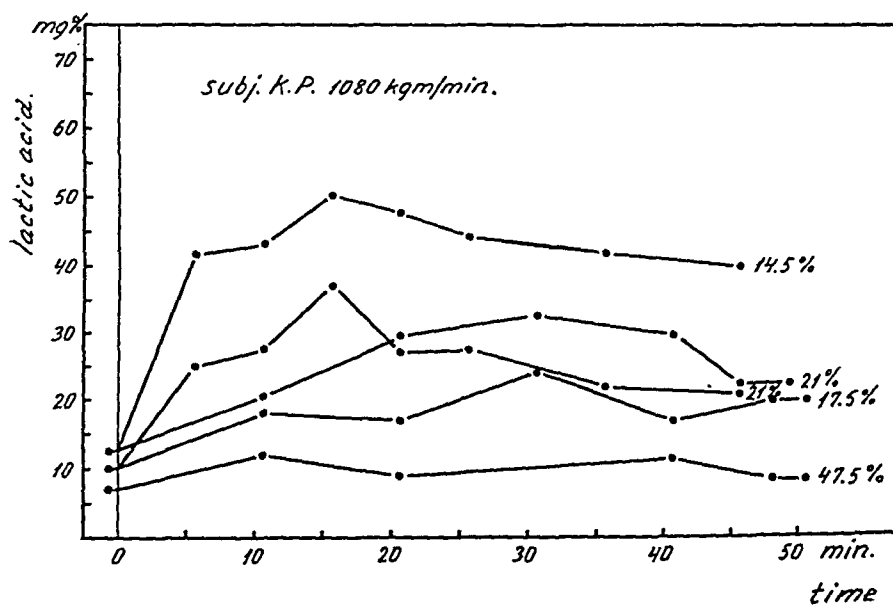


Fig. 2. Blood lactic acid of subject P. working 1080 kgm/min. with varying oxygen percentages in inspired air.

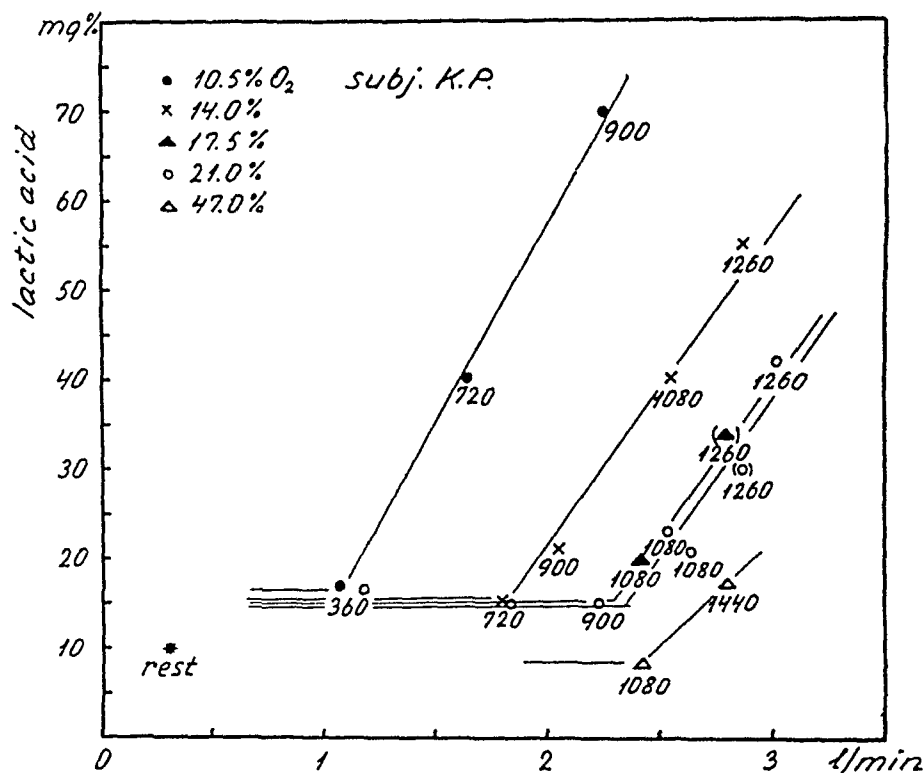


Fig. 3. Relation of blood lactic acid in steady state to oxygen consumption with varying oxygen percentages in inspired air. Subject P.

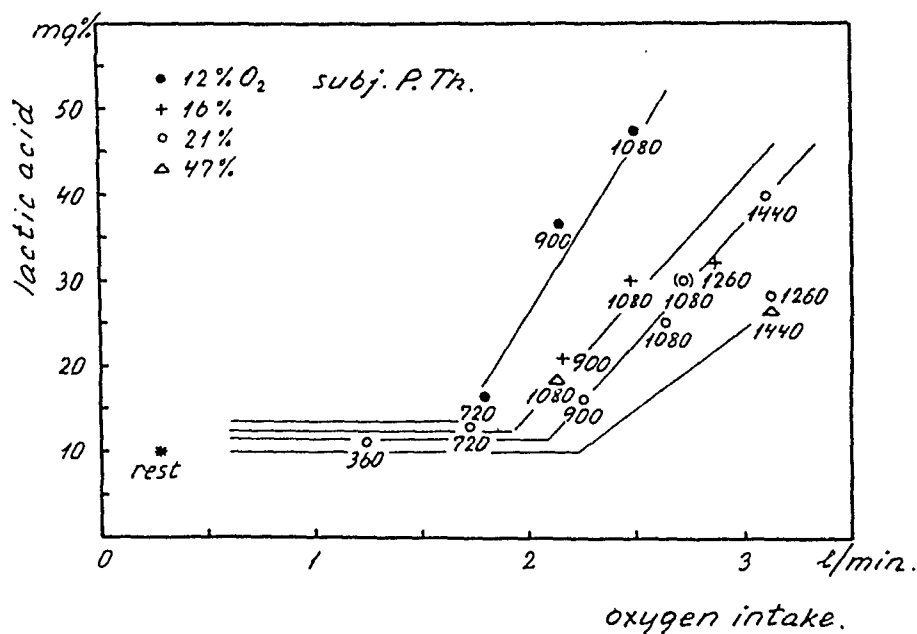


Fig. 4. Relation of blood lactic acid in steady state to oxygen consumption with varying oxygen percentages in inspired air. Subject Th.

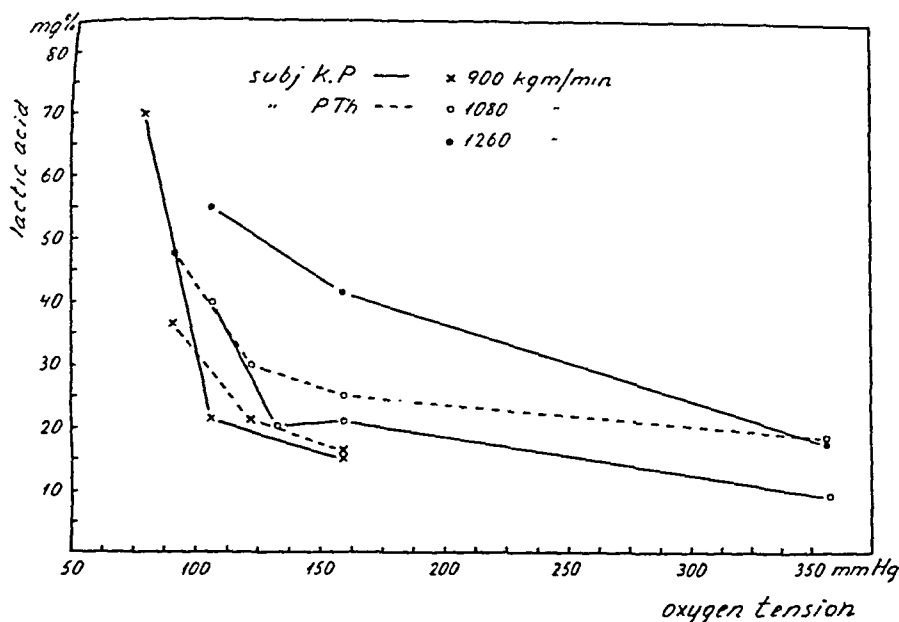


Fig. 5. Relation of blood lactic acid in steady state to oxygen tension in inspired air at varying grades of work.

Figure 1 shows the rises of lactic acid of subject P. when working 900 kgm/min. with different oxygen-nitrogen mixtures, figure 2 when working 1,080 kgm/min. During the period of the experiments the subjects' standard of training was slightly improved. This fact had an influence on the lactic acid rise of subject P. at grades of work higher than 1,080 kgm/min., on that of subject Th. at grades higher than 900 kgm/min., but in both cases the extra rise during anoxemia was unchanged. The respiration of 10.5 % oxygen mixtures had a marked confusing effect on the mind of subject P. Nevertheless he could perform, in the experiment of May 27th, a work of 900 kgm/min. for 50 min. with a lactic acid concentration of 70 mg %, and with a subjective feeling of doing it without exhaustion. In figures 3 and 4 the steady state values of lactic acid are plotted on the ordinate with the corresponding oxygen consumption values on the abscisse. In figure 5 the steady state values of lactic acid for a given grade of work are plotted on the ordinate with the inspiratory oxygen partial pressure on the abscisse. Figure 5 shows that there does not exist a simple inverse proportionality between the extra rise of lactic acid and the decrease of inspiratory oxygen partial pressure. When the pressure decreases towards 75 mm Hg the extra rise of lactic acid is steeply increased.

Discussion.

An important fact is that the oxygen consumption for a given intensity of work is independent of the inspiratory oxygen partial pressure within certain limits. In spite of a reduced oxygen percentage of the inspired air the organism secures an oxygen consumption sufficient to prevent progressive exhaustion and to ensure the reaching of a steady state of blood lactic acid. The ventilation is increased, giving a relative increase of alveolar oxygen pressure, and the minute volume of the heart is probably increased during these experiments with a sudden anoxemia, compensating the reduced oxygen saturation of the hemoglobin (ASMUSSEN and CONSOLAZIO 1941). This regulation is caused by the anoxia and probably also by anaerobic decomposition products other than lactic acid from the working muscles (ASMUSSEN and NIELSEN 1946). The fact that the rise of blood lactic acid from the rest value to the steady state value is significantly higher at low oxygen pressures than at a normal oxygen pressure can be regarded as a sign of a relatively greater anoxia in the working muscles, causing an increased anaerobic carbohydrate decomposition. This relatively greater anoxia is then caused by the reduced oxygen pressure of the arterial blood, slowing the delivery of oxygen to the muscle tissues, and probably by an insufficient blood flow to these active tissues. The lactic acid which accumulates in the organism during work is partly resynthesised to glycogen, probably to the greatest extent in the liver, partly oxidised, probably by all tissues. This oxidation of lactic acid and perhaps of other anaerobic products in the tissues during the steady state of lactic acid level will thus cause an oxygen consumption corresponding to the reduction in oxygen consumption of the partly anaerobically working muscles. The total oxygen consumption of the organism will thus be the same with high or low oxygen pressures in spite of the state of relatively greater anoxia in the active muscles during experiments with low oxygen pressures.

From this standpoint the effect of training on the blood lactic acid level during work may be explained as the result of an increased blood flow to the working muscles.

About the cause of the acclimatisation process of lactic acid during work, EDWARDS states the following (1936): "the inability

ot accumulate large amounts of lactic acid at high altitudes suggests a protective mechanism preventing an already low arterial saturation from becoming markedly lower by shift of the oxygen dissociation curve through acid effect. It may be that the protective mechanism lies in an inadequate oxygen supply to essential muscles, *e. g.* the diaphragm or the heart." This explanation is not supported by the results of work experiments on subjects with lowered bicarbonate concentrations of the blood caused by an administration of H_4NCl per os, an artificial acidosis (ASMUSSEN, NIELSEN and v. DÖBELN 1946). These experiments showed that a slight degree of acidosis did not influence the rise in concentration of blood lactic acid after maximal grades of work of short duration, nor the capacity for work. The experiments also showed that the capacity for maximal work of short duration was reduced if the subjects breathed nitrogen-oxygen mixtures with a low percentage of oxygen, but that the lactic acid peak after exhaustion was independent of the oxygen percentage. That means that the formation of lactic acid per unit of performed work was higher the lower the oxygen percentage.

Thus a moderately lowered bicarbonate concentration of the blood does not seem to influence the ability of a subject to stand high concentrations of lactic acid. Probably the effect of acclimatisation to low oxygen pressure on lactic acid formation during work is caused by the same processes as the effect of training. The apparently lowered capacity to accumulate blood lactic acid after acclimatisation is then caused by circulatory failure perhaps in the central nervous system independently of the blood lactic acid level.

The extra rise of lactic acid during work with a low oxygen pressure may also be explained as due to a relatively reduced assimilation rate of lactic acid because of anoxia in other tissues than the active muscles. The dissimilation rate will increase with increasing blood lactic acid levels, explaining why a steady state is reached. COLLEDAHL (1943) showed that during severe attacks of experimental asthma bronchiale in guinea-pigs the blood lactic acid level is increased, while the oxygen consumption is reduced in most tissues and especially in the liver. It is possible that an inhibited resynthesis of lactic acid to glycogen in the liver plays a part in the cause of the extra rise of lactic acid during anoxia.

It has been shown above that the extra rise of lactic acid has not a simple inverse proportional relation to the inspiratory oxygen

partial pressure (figure 5), but is steeper when the oxygen pressure sinks down towards 75 mm Hg. This can be explained by the further fact that when the inspiratory oxygen pressure, and therefore also the alveolar oxygen tension, is very low, the corresponding arterial oxygen saturation of the hemoglobin, as read on the oxihemoglobin dissociation curve, will be read on the steep part of this curve. A small decrease of oxygen tension thus causes a relatively great decrease in arterial hemoglobin saturation, with a relatively much increased anoxia in the working muscles as a result.

The authors wish to express their gratitude to Professor EM. HANSEN, D. Phil., and Professor P. BRANDT REHBERG, D. Phil., for extending to us the facilities of the laboratories for the theory of gymnastics and for zoophysiology, Copenhagen, and also to Mr. E. ASMUSSEN, D. Phil., who suggested this work, and Mr. M. NIELSEN, D. Phil., both of whom were in many ways of great help.

Summary.

The concentration of blood lactic acid was studied in subjects working on a bicycle ergometer and breathing nitrogen-oxygen mixtures with a varying percentage of oxygen, with special reference to the conditions when the work had been continued long enough to ensure the reaching of a steady state of blood lactic acid. The rise from the rest value to the steady state value was found to have a certain inverse proportional relation to the partial pressure of oxygen of the inspired gas mixture. The causes of this fact, and of the effect of acclimatisation on blood lactic acid levels during work as investigated by EDWARDS, are discussed.

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Protein Metabolism of Tissue Cells in vitro.

5. The Accessory Growth Substances in Sugar-free Malt Extracts.

By

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In recent years we have been studying some dialysable substances acting as accessory growth substances for tissue cells in vitro (FISCHER 1941, 1942) and which are found in extracts from yeast and barley malt (ASTRUP, FISCHER and VOLKERT 1945, ASTRUP and FISCHER 1945) as well as in animal materials. In our latest paper (ASTRUP and FISCHER, 1946) we mentioned difficulties encountered in the purification processes, which were to some extent due to the large sugar content of the solutions. We have hesitated to remove this sugar, which for the most part must be assumed to be maltose, by a fermentation with living yeast, as we feared to interfere with the content of accessory growth substances in the solutions, either by removing active substances from the solutions or by introducing new ones. As, however, it was not found possible to remove the sugars by simple chemical means we tried the fermentation, and we succeeded in this manner in removing the sugars, as a rule without interfering with the accessory growth substances. It must be pointed out, however, that in some cases crude extracts yielded almost completely inactive solutions after the fermentation, without it being possible to disclose the reason for this. The fermentation process is therefore not very suitable as a routine procedure for the purification of the substances.

It is the purpose of this paper to describe the properties of the accessory growth substances as present in these fermented solutions, with which it was possible to make some preliminary experiments on the importance of different sugars for the growth of animal tissue cells. Some of the results obtained so far have been mentioned recently (FISCHER 1946).

Experimental.

The action of the active substances was investigated in the manner described in previous papers, using chicken heart fibroblasts placed in dialysed media. The sugar content of the solutions was determined by the method of HAGEDORN and JENSEN (1923), and the results conventionally expressed as the corresponding amount of glucose, even if maltose is concerned and the substances responsible for the remaining reduction in the fermented solutions are unknown.

I. Crude Extract. Method III.

Previously we prepared our crude extracts by treating the malt with water at about 35°. It was now found that lowering this temperature decreased the content of sugar without interfering with the content of active substances. A crude extract (V—287.1) made after Method I, ASTRUP and FISCHER (1945), at 35° thus contained 32.6 mg sugar per ml, while a similar extract made at room temperature (V—287.2) contained 18.6 mg sugar per ml. Further lowering of the temperature did not decrease the sugar content further (V—397), and only minor differences were found when the acidity during the extraction was changed from the natural pH about 5.9 to pH 4.0, 5.5, 7.0, 8.0 or 9.75 (V—287, V—366). This amount of sugar must therefore be present as such in the barley malt, while the rest is formed during the extraction period at 35°. By lowering the temperature the nitrogen content is also decreased.

There seems to be no difference in activity between the solutions prepared at room temperature either by precipitation of the supernatant liquid after the extraction (method I) or by directly pouring alcohol on the mixture of malt and water (method II, ASTRUP and FISCHER, 1946). There was no difference in the

content of sugar in these two cases and only small differences in the contents of dry matter and nitrogen (*V*—307). For our further investigations we therefore as a rule prepared an extract of malt as in Method II, but at room temperature.

Preliminary experiments with fermentation by means of bakers yeast showed that after about 48 hours at room temperature (about 20°) the fermentation stopped, leaving a residual reduction corresponding to about 3—6 mg glucose/ml. The nitrogen content showed only small variations and the activity of the solutions usually seemed identical with that of solutions not fermented. The fermentation of a synthetic medium of the composition commonly used for cultivating yeast introduces no active substances in the solution (*V*—284). The following method was therefore devised for preparation of a fermented crude extract:

800 g of ground and sifted barley malt (pilsner malt dried at 90°) was stirred 1—2 hours at room temperature with 2 l of water. Then 61.96 % ethyl alcohol was added and after standing until the next day the mixture was filtered on a Büchner funnel. The filtrate was evaporated in vacuo (water bath at 40—50°) until all the alcohol was removed. The watery solution was poured from the lipids precipitated during the evaporation and filled up to the original volume (2 l). Then 80 g of bakers yeast was added (paying no regard to the amount of suspended matter (lipids) in the solution) and the mixture was placed at 20—25° with slowly mechanical stirring for 48 hours. The yeast was removed by centrifugation on a small separator of the continuous type and leaves about 1,800 ml crude extract. It contains 3.98 mg sugar and 0.33 mg N per ml (*V*—321). The content of non-fermentable sugar and nitrogen varies with the different samples. Activity test see Fig. 1.

2. Stability of the Fermented Solutions.

The stability of the active substances as present in the fermented crude extracts was investigated in order to compare with previous results obtained with the sugar containing solutions, as it is known that the presence of sugars may influence to a large extent the stability of labile substances.

By heating at acid and alkaline reaction the solutions only become very slightly coloured (yellow and light brown), while the previous sugar containing solutions yielded very dark solutions, especially at alkaline reaction. At the same time the stability of the active substances to heat is found to be very much increased. Heating at pH 3 in a boiling waterbath for 2 hours seems not to

influence the biological action much, and heating in the same manner at pH about 8.5 does not completely destroy the activity. Also treatment with N_2O_3 in dilute acetic acid does not result in inactive solutions (V—294, V—308).

Under such conditions therefore the active substances seem to be much more stable than we have hitherto assumed. Either the sugars contained in previous extracts combine during the heating

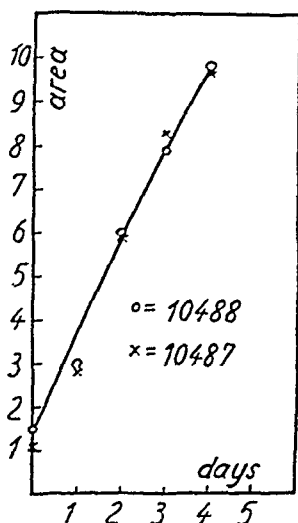


Fig. 1. Comparison between a crude extract prepared without fermentation (culture no. 10487) and after fermentation (no. 10488). Exp. V—313.

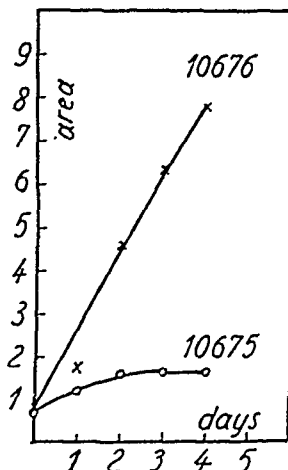


Fig. 2. Supernatant from alkaline precipitation with ethyl alcohol, V—320.2 (culture no. 10675). Precipitate, V—320.3 (culture no. 10676).

with the active substances, or, more probably, the heating of the sugars with acids or alkalis result in the formation of products toxic to the tissue cultures, thus disturbing the action of the active substances.

3. Alkaline Precipitation with Ethyl Alcohol.

The fermented extract prepared as described may be concentrated in vacuo to a very small volume before a sirupy consistence is obtained, and this solution can be made alkaline and precipitated with alcohol with better results than previously, as practically no activity is left in the alcoholic solution.

A fermented extract (1,100 ml) made from a sample of malt from which the husk was not completely removed, containing 1.125 mg N/ml and 7.86 mg sugar/ml, was evaporated in vacuo to a thin sirup

and made alkaline to phenolphthalein with 2-n NaOH. The resulting solution (37.5 ml) was precipitated with 6 vol. of ethyl alcohol (225 ml) and stored at 0° till the next day. The clear supernatant was removed by decantation, diluted with water, neutralized and the alcohol removed by evaporation in vacuo. After dilution to the original volume and filtration it contained 0.43 mg N and 2.44 mg sugar per ml (*V*—320.2).

The alkaline precipitate was dissolved in water and neutralized. After dilution to the original volume and filtration it contained 0.55 mg N and 5.10 mg sugar per ml (*V*—320.3). While all the sugar is retained in the solutions, some nitrogen is lost in the small amount of precipitate removed by filtration. Fig. 2 shows a comparison between these two solutions. It is seen that the alkaline alcoholic solution is completely inactive.

4. Precipitation with Glacial Acetic Acid and Ethyl Alcohol.

After the removal of the sugars it was possible to carry out the fractionation with glacial acetic acid and ethyl alcohol in much more concentrated solutions than previously used. The absence of the sugars further facilitated the fractional precipitation to such an extent, that it was possible to isolate an active middle fraction as a relatively non-hygroscopic powder, which could be stored without loss of the biological action.

A solution (*V*—334) corresponding to 1,600 ml original crude extract (Method III) and which had been precipitated at alkaline reaction was evaporated in vacuo to a sirup and dissolved in 100 ml glacial acetic acid at 40°. Then 50 ml abs. alcohol were cautiously added, and the mixture left standing at room temperature overnight. The liquid was removed by decantation and centrifugation and the precipitate dissolved in water. After dilution to the original concentration it contained 1.04 mg sugar and 0.043 mg N per ml and is inactive (*V*—334.2). To the supernatant 450 ml abs. alcohol was added and a fine, crystalline precipitate separated. After standing for some hours at room temperature it was removed by means of a glass filter, dissolved in water and evaporated in vacuo. After diluting to the original volume it contained 0.95 mg sugar and 0.137 mg N per ml (*V*—334.3).

The filtrate from *V*—334.3 was evaporated in vacuo with water. After dilution to the original vol. it contained 0.83 mg sugar and 0.156 mg N per ml.

The biological test is shown in fig. 3, from which it is seen that almost all the activity is retained in the middle fraction *V*—334.3.

The precipitate separated in the middle fraction may without much loss of active material be treated on the filter with abs. alcohol and dry ether and dried in a vacuum desiccator over conc. H_2SO_4 and NaOH. After transformation to a dry powder it lost most of its hygroscopic

properties, $V-335.3$. From 1,600 ml original solution 6.90 g was obtained, corresponding to 4.30 mg per ml original solution. This preparation contains 8.8 % ash (as sulphate), 33 % sugar (1.39 mg per ml), 4.1 % N (0.177 mg N per ml) and 6 % pentose (determination after HOFFMANN, 1927). It reacts only slowly and to a small extent with Benedict's reagent, but reduces large amounts of permanganate in

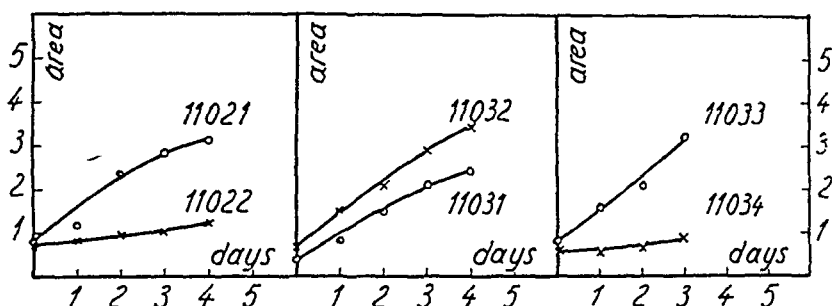


Fig. 3. Original solution $V-334.1$: culture no. 11021, 11031 and 11033. First precipitate $V-334.2$: culture no. 11022. Middle fraction $V-334.5$: culture no. 11032. Mother liquid $V-334.4$: culture no. 11034.

alkaline solution (carbonate). After hydrolysis with 0.1-n HCl at 100° in 15 min. the sugar content is only increased some 15 %.

Later it was found that the sticky precipitate obtained after the alkaline precipitation with alcohol could be treated directly with glacial acetic acid leaving an almost inactive precipitate, because most of the water was absent at this point.

3,600 ml of a fermented malt extract (containing 5.10 mg sugar and 0.745 mg N per ml) was evaporated to dryness in vacuo, dissolved in 70 ml water and 25 ml 2-n NaOH (alkaline to phenolphthalein) and precipitated with 900 ml 96 % ethyl alcohol (6 vol). After standing over night at 0° it was decanted and 200 ml glacial acetic acid added to the precipitate and left standing at room temperature until the next day. It was then heated in a water bath at about 70°, whereby most of it is dissolved, cooled under tap water and left until next day at room temperature. The supernatant (205 ml) was removed by decantation and precipitated with 5 vol. of abs. alcohol (1,025 ml). After standing for a few hours the precipitate may be isolated as a dry powder as previously described ($V-272$). It contained most of the biological activity of the original solution. Calculated on the original solution it contained 1.02 mg sugar and 0.136 mg N per ml.

5. Other Purification Experiments.

In accordance with the purification procedure previously used we now tried an extraction with 90 % phenol, but without success

as the activity was found partly in the water layer and partly in the phenol layer, while previously only the water phase was active. Probably the more purified and concentrated solutions now used were the cause of this failure.

Precipitation of a fermented extract with neutral lead acetate removes inactive material containing about half the amount of nitrogen but almost no sugar, and treatment with mercury sulphate in diluted sulphuric acid removes a further nitrogen containing, inactive precipitate (*V*—310, *V*—312, *V*—313, *V*—315). After a preliminary purification with glacial acetic acid, as described, precipitation with lead acetate presented difficulties (*V*—340, *V*—341) and was abandoned. Precipitation with silver acetate at varying acidities was tried but without success (*V*—385, *V*—388). Also the removal of the remaining reducing substances after the method of VAN SLYKE (1917) for the elimination of sugars did not result in a separation of inactive material from active, even if most of the sugars remained in the precipitate (*V*—313, *V*—315).

6. Other Properties of the Sugar-free Extracts.¹

Using the yeast-treated malt extracts it was now possible in some experiments to show the importance of sugars for the maintenance and growth of tissue cells.

When the dialysis of the plasma, serum and embryonic extract used for cultivating the tissues was carried out against Ringer solution without our usual addition of glucose, and a Tyrode solution containing no glucose was used in the culture work, the tissue cells were unable to survive when placed in such glucose-free media. This was the case even in the presence of fermented and purified malt extract, containing a residue of non-fermentable substances reacting with ferricyanide under the conditions of the Hagedorn and Jensen determination. These substances, which probably may be non-fermentable sugars, are therefore unable to replace glucose in the nutrition of the cells. When glucose was added together with these malt extracts the tissue growth was that usually obtained by adding the malt extract to the glucose containing media (*V*—320, *V*—326).

In this manner it was possible for us to study the action of different sugars on the growth of tissue cells, and it was found

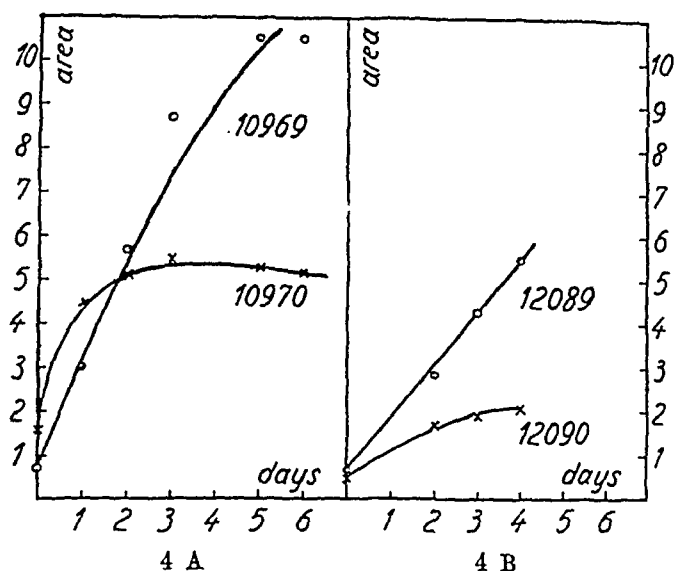


Fig. 4 A and B. A. Action of purified sugar-free malt extract (V-326.1) in the presence (culture no. 10969) and in the absence of glucose (culture no. 10970). B. Action of a purified extract (V-324.1) in the presence of 0.1 % glucose (culture no. 12089) and of 0.1 % xylose (culture no. 12090).

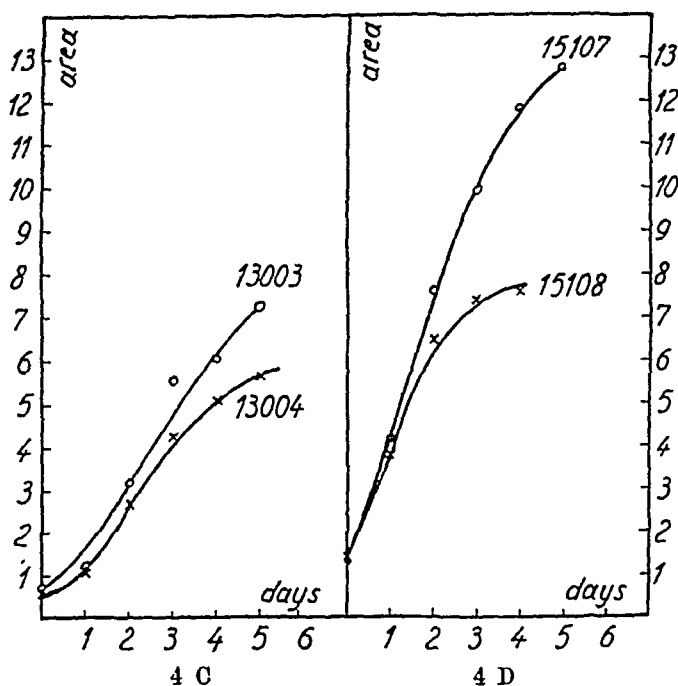


Fig. 4 C and D. C. Action of V-324.1 in the presence of 0.1 % glucose (culture no. 13003) and of 0.1 % fructose (culture no. 13004). D. Action of V-324.1 in the presence of 0.1 % glucose (culture no. 15107) and of 0.1 % mannose (culture no. 15108).

that fructose (V—344, V—361) and mannose (V—367) were able completely to replace glucose. Some action was also shown for galactose (V—368) and probably also for maltose (V—360, V—381). The following were found inactive: saccharose, lactose, xylose, arabinose, glycogen (liver) soluble starch, α -glycerophosphate, pyruvic acid, d-lactic acid and glucosamine. We have here a new confirmation of the importance of the glucolysis (or fructolysis) for the metabolism and growth of tissue cells. Some examples are seen in Fig. 4.

In this connection it is interesting to note that according to MANN (1946) spermatozoa are able anaerobically to utilize equally well fructose, glucose and mannose, while WARBURG, POSENER and NEGELEIN (1924) for the anaerobic glycolysis of tissue slices found the highest values for glucose and mannose; fructose and galactose only yielding a fraction of those values. According to DICKENS and GREVILLE (1933), most tissues under anaerobic conditions are unable to metabolize fructose with a reasonable velocity, cfr. also ROSENTHAL (1930).

In our case it is not possible to obtain quantitative data about the ability to utilize fructose instead of glucose, but no considerable difference was observed. Even if the presence of oxygen (air) in the Carrel culture flasks allows of oxidative metabolic processes, the importance of these sugars together with the formation of lactic acid during the cultivation, cfr. DEMUTH and MEIER (1929) and WILSON, JACKSON and BRUES (1942), points to glucolysis (or fructolysis) as the deciding process for the growth of the tissue cultures. It is also known that tissue cultures may be grown to some extent under anaerobic conditions, WIND (1926), LIPMANN (1933), LASER (1933), FISCHER, FOGH and BOHUS JENSEN (1945).

Summary.

1. The accessory growth substances present in barley malt extracts were purified by means of fermentation with bakers yeast, alkaline precipitation with ethyl alcohol and precipitation in glacial acetic acid with ethyl alcohol. A dry powder containing most of the activity could be prepared. The active substances in the fermented solutions are considerably more stable than previously assumed.

2. Using such sugar-free preparations it was possible to show

the ability of the tissue cells to utilize glucose, fructose and mannose for growth purposes. Without the addition of the malt extracts the sugars were inactive.

This work was aided by a grant from "*Teknisk-kemisk Fond*". The barley malt was placed at our disposal by the *Carlsberg Breweries* and A/S "*Ferrosan*", Copenhagen, has aided us in the preparation of crude extracts.

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The Effect of Adenosine Triphosphate and Related Compounds on the Perfused Superior Cervical Ganglion.

By

ANDERS LUNDBERG.

(Received 21 December 1946.)

Recently it has been demonstrated that adenosine triphosphate (ATP) elicits contractions in the normal and denervated striated muscle, as well as in smooth muscle (BUCHTHAL, DEUTSCH and KNAPPEIS, 1944; BUCHTHAL and FOLKOW, 1944; BUCHTHAL and KAHLSON, 1944, 1946). On the other hand ATP has no effect on small bundles of cardiac muscle (DEUTSCH and LUNDIN, 1946).

In the last few years it has also been shown that ATP is of great importance for the synthesis of acetylcholin (NACHMANSOHN and MACHADO, 1943; FELDBERG and MANN, 1945; FELDBERG and HEBB, 1945).

It appears that ATP also stimulates the sympathetic ganglion, and this paper aims at investigating this effect. During the course of our experiments we were informed by FELDBERG that he and HEBB were dealing with the same problem (results published 1946). They have shown that ATP and creatine phosphate stimulate the ganglion, and that the effect was obtained 8—9 days after cutting the preganglionic cervical sympathetic nerve. It is known that the capacity of the ganglion to synthesize acetylcholin is lost after that time and they conclude that the stimulating effect of ATP and creatine phosphate are not dependent on the acetylcholin metabolism of the preganglionic endings.

Method.

The experiments were performed on the perfused superior cervical sympathetic ganglion of the cat with the retraction of the nictitating membrane indicating stimulation of the ganglion cells. The ganglion was perfused as described by KIBJAKOW and by FELDBERG and collaborators, but instead of hydrostatic a pulsatile pressure was employed. The cats were under chloralose, and evipan or ether was used as pre-

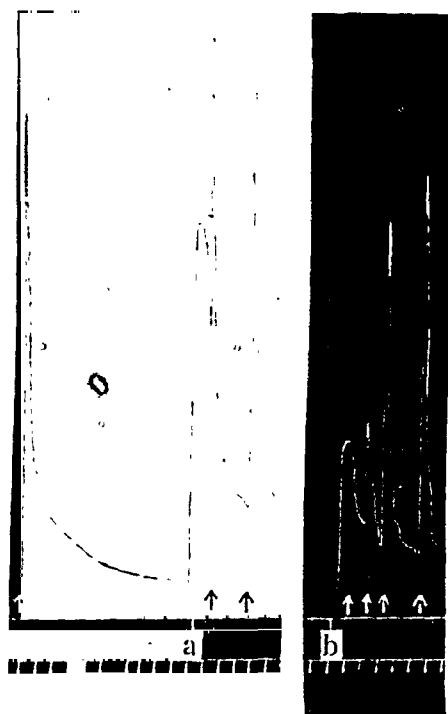


Fig. 1. Retraction of the nictitating membrane after injection in the perfused ganglion of a) 0.5 mg ATP b) 2 mg sodium citrate. At arrows electrical stimulation during 10 sec. of the preganglionic sympathetic nerve. Time in min.

narcotic. In some of the experiments the blood flow was maintained through the ganglion. The ganglion was prepared in the usual manner, but, instead of perfusion with tyrode solution, a piece of the common carotid artery was replaced by a rubber tubing, through which the blood flow to the ganglion passed. In the rubber tubing injections could be made as close arterial injections, after the rubber tubing central to the site of injection had been occluded. The disadvantage of this procedure is that the slow blood flow through the ganglion often ceases completely after about an hour.

Oxygenated Tyrode solution was used as perfusion fluid. In some of the experiments Dextran was added, in order to ensure a suitable colloid osmotic pressure (GRÖNVALL and INGELMANN, 1945).

The preganglionic fibres were stimulated by a Tyratrone stimulator and glass-shielded platinum electrodes were applied.

The following substances were used, most of them kindly supplied by Drs BUCHTHAL and DEUTSCH.

ATP: Injected as sodium salt. Preparation described by BUCHTHAL et al. 1944. The analysis of a typical specimen gave a total P of 5.87 mg/ml; 7' P 3.98 mg/ml; P_o: 0; the solution containing 31 mg ATP/ml.

Adenosine: The substance was prepared from yeast nucleic acid by the method of Brederick.

Adenylic acid (muscle-): Injected as sodium salt. The analysis gave a total P of 0.20 %; inorganic P 0.005 %; inorganic P + P hydrolysable in acid 0.15 %.

Sodium triphosphate: the substance contained c. 85 % sodium triphosphate and 12 % sodium ortophosphate.

Sodium pyrophosphate, sodium ortophosphate, sodium citrate and sodium oxalate were of analytical purity. All substances were administered in iso-osmotic solutions, and with pH adjusted to 7.3.

Results.

The following effects were obtained on injection of the substances tested on the ganglion perfused with Tyrode. The injected amounts were 0.1 to 0.2 ml.

ATP stimulates the ganglion cells, the threshold dose being 0.1 to 0.3 mg calculated as free acid. Our ATP was prepared over the Ba-salt, and, in spite of careful purification, small amounts of Ba-ions might have been present. Ba-ions have a sensitizing effect on the ganglion cells (unpublished observations), but the concentration of Ba-ions in the preparation used was not sufficient to cause anything but a slight sensitization.

Adenosine: no action of this substance could be demonstrated. Greatest amount tested 2.4 mg.

Adenylic acid stimulated the ganglion, but only in high doses such as 2 mg.

Sodium triphosphate and sodium pyrophosphate also stimulate the ganglion. In some experiments the threshold dose was lower for triphosphate, but in most of the experiments it was almost the same for these substances amounting to about 0.5 to 0.75 mg.

Sodium ortophosphate had the same effect as the other inorganic phosphate compounds tested, but only in higher concentration. Only two experiments were performed, and 1.5 mg had to be injected before retraction of the nictitating membrane was observed. The results are qualitatively the same as those

found by FELDBERG and HEBB, who however did not report the effect of sodium ortophosphate.

Ca-ATP is hardly soluble and theoretically the effect of ATP can be explained by the withdrawal of Ca-ions from the synapse.

HARVEY and MACINTOSH have shown that if the ganglion is perfused with Ca-free Locke solution the ganglion cells discharge and the synaptic transmission is blocked or depressed. BRONK, LARRABEE, GAYLOR and BRINK came to the same results after perfusion of the stellate ganglion with sodium citrate. ATP, however, stimulates the ganglion in amounts which are smaller than those of sodium citrate or sodium oxalate necessary for producing the corresponding effect. In one experiment shown in fig. 1 almost maximal stimulation was obtained with 0.5 mg of ATP, whereas the threshold dose of sodium citrate in this experiment was unusually high, 1 mg. The second manifestation of Ca-lack in the ganglion, blocking or depression of the synaptic transmission, is not regularly observed when such small amounts as 0.1 ml are injected, containing iso-osmotic solution of the Ca-precipitating substance. But with slow perfusion, either after some hours' perfusion with saline or when the blood flow through the ganglion is maintained, a short block or depression of the synaptic transmission is observed after injection of 0.1 ml iso-osmotic solution of sodium citrate (after sodium oxalate a protracted block is observed, probably a toxic effect). In fig. 1 an experiment is shown in which ATP does not depress synaptic transmission, whereas sodium citrate in an amount which is less stimulant, causes a short block and a somewhat longer depression. Thus there is evidence for the hypothesis that ATP stimulates the ganglion by a mechanism other than the withdrawal of Ca-ions.

As regards sodium triphosphate and sodium pyrophosphate, it is much more difficult to decide whether the action of these substances is dependent on their capacity to withdraw Ca-ions. The Ca-salts of these polyphosphates are less soluble than Ca-ATP. Sodium citrate and sodium oxalate in similar concentrations have the same stimulating effect. With slowly perfused ganglions, the injection of sodium triphosphate or pyrophosphate causes a depression of the synaptic transmission. Though this depression is sometimes less than that caused by an amount of sodium citrate which has the corresponding stimulating effect, the possibility can not be excluded that these anorganic polyphosphate compounds may act in their capacity of Ca-precipitating substances.

Further investigation of this problem is necessary and we hope to be able to return to it later on. Finally as regards the few experiments with sodium ortophosphate there is no reason to explain the stimulating effect by a mechanism other than the withdrawal of Ca-ions.

Summary.

ATP stimulates the sympathetic ganglion in concentrations from 0.1 to 0.3 mg. Adenylic acid, sodium triphosphate, sodium pyrophosphate and sodium ortophosphate also have a ganglion-stimulating effect, whereas adenosine has no such effect. The possibility that these substances act by withdrawing Ca-ions is discussed.

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From the County Hospital of Aalborg, Medical Department.

Some Determinations of the Total Body Water in Man by Means of Intravenous Injections of Urea.

By

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Received 16 January 1947.

Determinations of the volumes of the various liquid systems in the organism by administration of a known quantity of some substance which is supposed to be evenly distributed in the system in question, is a well known principle. From the concentration which the substance will attain it is possible to calculate the volume.

In this way the blood volume in animals and humans has been determined. Also the volume of the extracellular fluid has been the object of similar investigations; thus LAVIETES, KLINGHOFFER and BOURDILLON used administration of sucrose, sulphate and thiocyanate. The most suitable of these substances was thiocyanate, and values of 20—28 p. ct. were obtained.

The intracellular fluid cannot be determined directly, but must be calculated as the difference between the total body fluid and the extracellular fluid.

The total amount of water can be determined by administration of substances which penetrate the cells of the organism and are distributed according to the water content of the various liquid systems. Until now, few investigations of this kind seem to have been made.

It should be mentioned here that, in his investigations of the oxidation of alcohol, WIDMARK found that such concentrations were obtained, as if the amount of alcohol administered was dis-

solved in a certain proportion of the body, the volume of which was the body weight multiplied with a factor called r . This volume of distribution of alcohol is generally considered to represent the total water content of the organism, and, according to WIDMARK, r is on an average 0.55 in women and 0.68 in men.

Later HOLST found by corresponding investigations that glycerol is distributed in a similar way as alcohol, and the constant r was found to be 0.45—0.62, average figure 0.52.

PAINTER has found by experiments on eight dogs that intravenously injected urea was distributed over an average of 63.0 p. ct. of the weight. 200—350 mg urea per kg was injected, and the blood urea was determined before and repeatedly after the injection. Diffusion equilibrium was obtained after one hour. By extending the curve to the moment of injection the difference of concentration was found, according to which the volume of distribution was calculated.

DANOWSKI used thiourea in corresponding experiments with dogs and found values of 65—85 p. ct.

In the following report some attempts to calculate the total volume of body water by means of intravenous injection of a known amount of urea are recorded. The experiments have exclusively been carried out on humans, mainly patients in this department, and a special method of calculation, adapted for clinical purposes, has been used.

Method.

In the experiments a 30 p. ct. solution of urea in water has been used; the solution has been sterilized by filtering through a bacteria filter. Generally 50 ml (: 15 gr urea) is injected intravenously. The quantity is read as exactly as possible from the scale of the syringe. Immediately before the injection the experimental person has evacuated his bladder, and a sample of venous blood has been taken for the determination of urea. Later on blood samples are taken at certain intervals, and the urine is collected for each period.

The volumes of the urine are measured, and the urea concentration in blood and urine is determined by the manometric hypobromite method of VAN SLYKE and KUGEL. According to these authors the urea concentration in blood can be determined with the standard deviation of 0.2 mg nitrogen or 0.4 mg urea per 100 ml. Here ten analyses of the same blood sample have been made with the following results:

85.5	83.4	83.5	83.3	84.1 mg p. ct.
82.7	82.7	83.0	83.6	83.7 » »

The mean value was 83.54 mg p. ct., and the standard deviation ± 0.81 mg p. ct. or 0.97 p. ct. of the mean value.

As to the urine analyses, only 95 p. ct. of the urea is determined, and 96 p. ct. of the ammonia present in the urine is included in the result. In ten analyses of the same sample of urine the following values were found:

2.13	2.09	2.11	2.10	2.10 p. ct.
2.10	2.09	2.08	2.06	2.11 » »

The mean value was 2.097 and the standard deviation ± 0.019 p. ct.

In all experiments double values of the concentration of urea in blood and urine have been determined.

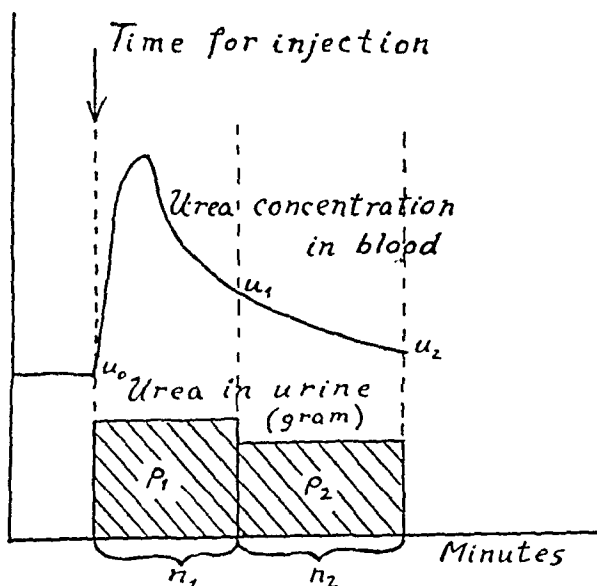


Fig. 1. Urea in blood and urine after intravenous injection of urea.

In the calculation of the volume of distribution of urea the special difficulty is encountered that an unknown amount of urea is produced in the organism during the experiment. In order to reduce this source of error the experiments have all been carried out in the forenoon after a protein-poor breakfast, so that the production of urea can be estimated to be especially low. Furthermore, it is assumed to be constant per unit time during the experiment.

If the volume sought is called V , and the blood urea at the beginning of the experiment and after the first and second period are called u_0 , u_1 and u_2 respectively, the total amount of urea in the body at the beginning of the experiment may be called Vu_0 . Now P gr urea is injected, and during the first and second periods p_1 and p_2 gr urea is excreted in the urine. During the same periods n_1x and n_2x gr urea is formed in the body, n_1 and n_2 designating the number of minutes in the periods and x the urea production per minute. Fig. 1 shows these

figures schematically. After the first period the total amount of urea in the organism is

$$Vu_0 + P - p_1 + n_1x = Vu_1 \dots \dots \dots (1)$$

and after the second period

$$Vu_1 - p_2 + n_2x = Vu_2 \dots \dots \dots (2)$$

From these equations V can be calculated:

$$V = \frac{n_2(P - p_1) + n_1p_2}{n_2(u_1 - u_0) + n_1(u_1 - u_2)} \dots \dots \dots (3)$$

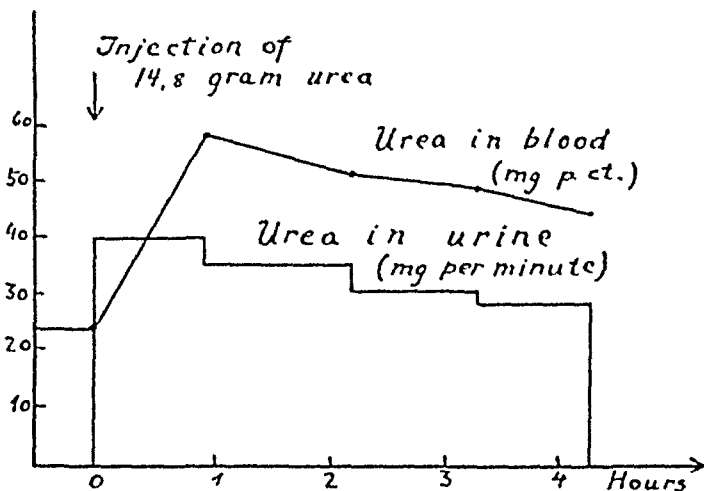


Fig. 2. Urea in blood and urine in a patient after injection of 14.8 g urea.

Usually the experiments have lasted four hours; the division into periods has been varied a little. Thus some of the experiments have been carried out in four periods of about one hour each and have thus made more than one determination of V possible.

The discomforts of the procedure have been rather moderate. In a few cases an aseptic thrombosis of a vein in the forearm has been observed after the injection and two of the experimental persons had a transient rise of temperature, but in both cases the solution used for the injection had been standing more than three weeks after the preparation.

Results.

In most cases it was found that one hour after the injection of 15 gr urea, the blood urea was 20—40 mg p. ct. higher than the starting level, after which it fell evenly during the following hours by about 2—5 mg p. ct. per hour (cfr. fig. 2). This is taken as an indication that sufficient equilibrium of diffusion of the urea injected is obtained after one hour.

In calculation of the volume of distribution of urea three values of blood urea are needed together with the excreted amount of urea in the two intermediate periods. In six of the experiments, in which four periods of one hour each have been used, three values for V have been calculated. The first period has been used in all cases and then the second period (a), second plus third period (b) or second plus third plus fourth period (c). Table 1 shows these values and the differences between them.

Table 1.

Three determinations of V in the same experiment.

Sex	Age	Height cm	Weight kg	V in the same experiment (liters)				Deviation from mean value (p. ct.)		
				a	b	c	mean value	a	b	c
♂	30	181	81.4	59.1	59.7	58.9	59.2	-0.2	+ 0.8	-0.5
	21	152	55.5	37.0	34.9	36.3	36.1	+ 2.5	- 3.3	+ 0.6
	19	180	79.5	56.5	57.1	57.5	57.0	-0.9	+ 0.2	+ 0.9
	27	176	70.0	57.1	56.7	57.4	57.1	0.0	-0.7	+ 0.5
	34	162	80.5	37.4	39.0	38.0	38.1	-1.8	+ 2.4	-0.3
	59	164	95.2	51.3	51.5	51.7	51.5	-0.4	0.0	+ 0.4

It will be seen that the greatest numerical deviation from the mean value was 3.3 p. ct. of the body weight. The average deviation was 0.91 p. ct. After this it has been chosen to make the first period of one hour and the second of three hours.

In this manner 16 persons in different states of nourishment, none of whom were obese, have been examined. The material consists partly of normal persons, partly of patients and convalescents in the department, in whom no disturbance of fluid balance was suspected. The results are recorded in table 2.

V was found varying from 64.5 to 82.5 p. ct. of the weight. This dispersion seems to some extent to be due to the persons' different heights and weights, which most plainly appears from fig. 3. Thus fat persons have a lower V than lean ones, and small persons seem to have a lower V than bigger ones in the same state of nourishment. An attempt has therefore been made to produce a correction of these variations, and the result has been the following formula:

$$V \text{ (p. ct.)} = \frac{h - 43 - 0.032 (w - 33.2)^2}{1.50 - 0.00027 (w - 33.2)^2}$$

Table 2.
Volume of distribution of uræa in 16 "normal" persons.

Diagnosis	Sex	Age	Height cm	Weight kg	V experi- mentally		V cal- culated p. ct.	Devia- tion p. ct.
					lit.	p. ct.		
Normal	♂	30	181	81.4	58.9	72.3	73.2	+ 0.9
Normal	♂	21	152	55.5	36.3	65.4	68.2	+ 2.8
Normal	♂	19	180	79.5	57.5	72.3	74.2	+ 1.9
Normal	♂	27	176	70.0	57.4	81.8	79.0	- 2.8
Normal	♂	31	166	66.0	48.6	73.6	73.2	- 0.4
Asthénia	♀	51	168	44.1	36.4	82.5	82.6	+ 0.1
Normal	♀	20	163	60.0	45.0	75.0	74.3	- 0.7
Poliomyelitis ant. ac. (con- valescent)	♂	18	169	54.7	44.0	80.4	80.9	+ 0.5
Sciatica	♂	20	170	61.0	49.8	81.7	79.6	- 2.1
Asthma bronchiale (con- valescent)	♂	34	178	67.7	54.5	80.5	82.2	+ 1.7
Hysteria	♀	28	158	47.5	34.9	73.5	75.1	+ 1.6
Neurosis cordis	♀	19	163	72.7	46.9	64.5	65.0	+ 0.5
Diphtheria (conval.)	♂	18	171	61.3	48.6	79.3	79.9	+ 0.6
Hysteria	♀	36	158	42.2	32.2	76.3	76.2	- 0.1
Sciatica	♂	44	166	55.0	42.0	76.4	78.9	+ 2.5
Asthma bronchiale (con- valescent)	♂	19	168	60.2	46.5	77.3	77.8	+ 0.5

in which h is the height in cm and w the weight in kg. This formula seems preliminarily to form a usable basis for the comparison of V when heights and weights are within certain limits. In the last columns of table 2 the values of V calculated in this way and their deviation from the experimental values are recorded. These deviations are all less than ± 3 p. ct. of the body weight.

In table 3 the results of eight experiments on patients suffering from adipositas are recorded. Here V was found to be from 40.3 to 58.9 p. ct. of the weight, a conspicuous difference from normal persons.

Table 3.
Volume of distribution of uræa in eight obese patients.

Sex	Age	Height cm	Weight kg	Over-weight		V.	
				kg	p. ct.	liter	p. ct.
♀	34	162	80.5	18.5	29	38.0	47.2
♀	69	160	80.6	20.6	34	39.7	49.2
♀	58	154	77.5	23.5	43	43.9	56.6
♀	45	158	84.4	26.4	45	49.7	58.0
♀	59	164	95.2	31.2	49	51.7	54.3
♀	53	156	87.0	31.0	55	39.9	45.9
♀	34	162	104.7	42.7	69	49.3	47.1
♀	44	172	140.5	68.5	94	56.7	40.3

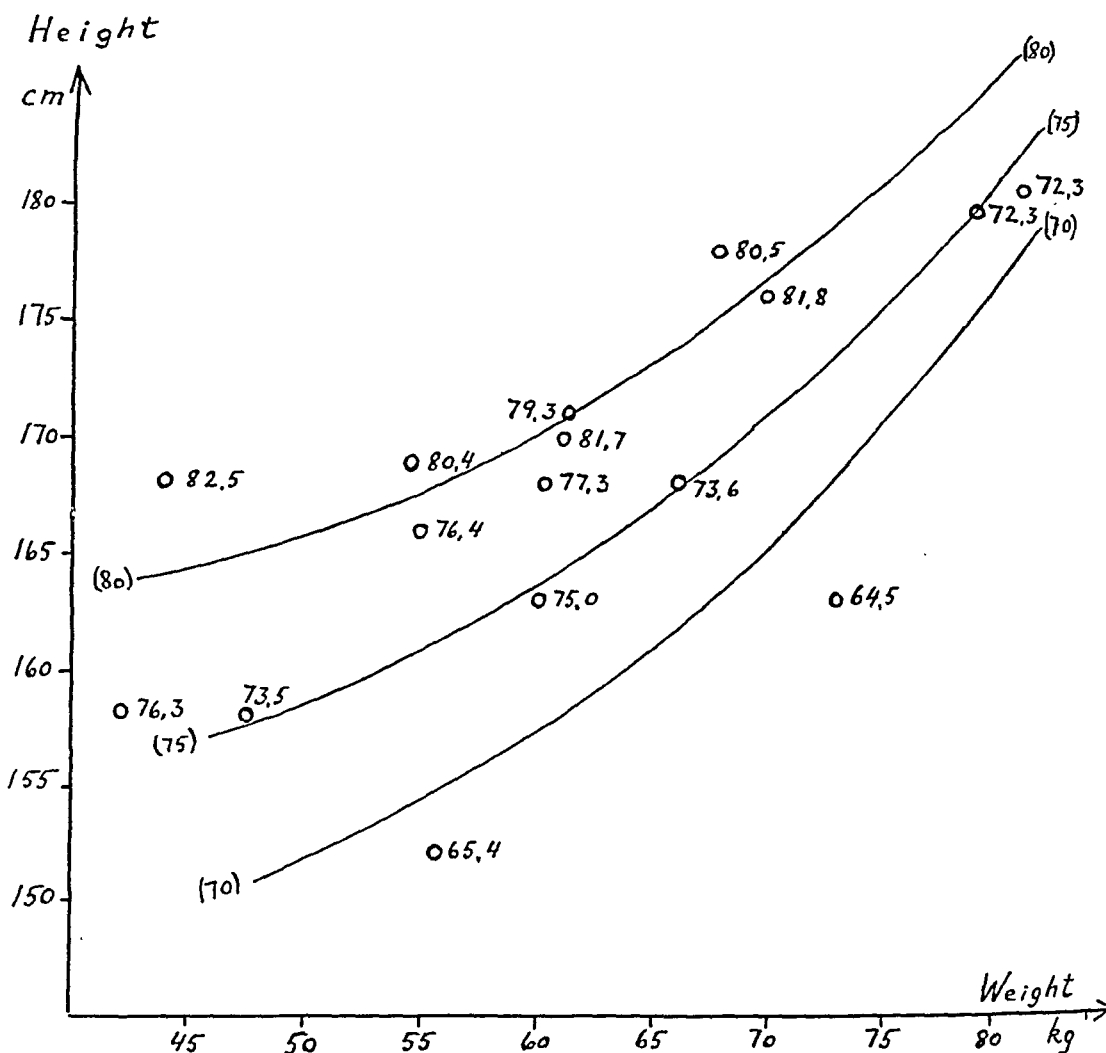


Fig. 3. Volume of distribution of urea (p. ct. of body weight) in 16 normal persons in relation to height and weight. The curves represent the equation

$$V = \frac{h - 43 - 0.032 (w - 33.2)^2}{1.50 - 0.00027 (w - 33.2)^2}$$

for V : 80, 75 and 70.

Discussion.

According to the results before us there can be no doubt that the injected urea is distributed over all the liquid systems of the normal organism, and that, to a great extent, it invades the cells so that it is probable that the volume of distribution of urea is a certain measure of the total water content of the organism.

In the analyses whole blood has been used, and in order to find the actual water content of the body the values for V found must be multiplied with the water concentration of the blood.

As WIDMARK also used whole blood for the alcohol determinations, the results can be directly compared with his constants r , which were on an average 0.55 in women and 0.68 in men. In the investigations before us the volume of distribution of urea in normal persons was on an average 75.8 p. ct. The difference may be due to a more complete diffusion of the urea into the cells, than is the case with alcohol, but it may also be due to different weight-to-height relationships in the two sets of subjects.

The possibility should also be considered that urea attains another and perhaps higher concentration in the cell water than in the intercellular water. In this connection it should be mentioned that RALLS most frequently found the urea concentration in the blood corpuscles a little higher than in the plasma, calculated in proportion to the amount of free water.

The hypobromite method is not the most exact method for determination of urea, but it is convenient for clinical purposes and can be carried out in most hospital laboratories. The error of the blood analyses is very insignificant and is partly to be found in the fact that the chemical reaction is not 100 p. ct. quantitative, partly therein that very small amounts of other nitrogenous substances, among others ammonia, are included in the determinations. As the question in these investigations is about *differences* in the urea concentration during four hours, the importance of systematic errors is further reduced.

As to the urine, the error is somewhat larger partly owing to the larger amount of ammonia in the urine, partly because only about 95 p. ct. of the whole amount of nitrogen is developed. However, it is only the difference between the urea output per minute in the two periods which is of any importance for the calculation. If the urea output is equal during the whole experiment, the exact amount of urea in the urine will be without interest, because the expression $n_2p_1 - n_1p_2$ is 0, and in almost all the cases this figure is much less than n_2P . Furthermore, it seems quite natural to include the ammonia of the urine in the analyses, as it is formed, for the major part, of the urea of the blood.

A condition for the reliability of the calculation is that the urea injected is not transformed or stored in the organism. As urea has hitherto been considered a waste product, there is no special reason

for assuming such a possibility, apart from the aforesaid formation of ammonia in the kidneys. But it should be mentioned in this connection that, in his experiments with thiourea, DANOWSKI found that this substance gradually "disappeared" in the body, the concentration in the blood decreasing faster than it could be expected, considering the amount of thiourea excreted in the urine, so that the calculated "volume of distribution" increased with the time passed after the injection.

Summary.

The author describes a method for calculation of the total water content of the human organism by means of intravenous injections of urea and determinations of urea in blood and urine. For these determinations the manometric hypobromite method of VAN SLYKE and KUGEL has been used. The volume of distribution of urea was found in normal persons varying from 64.5 to 82.5 p. ct. of the weight, and in obesity patients, from 40.3 to 58.9 p. ct. The variations in the normal subjects seem to be related to the different sizes and state of nourishment of the persons investigated.

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Glucose Tolerance of Partially Hepatectomised Rabbits.

By

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In their monograph "The Physiology of the Impaired Liver" BOLLMAN and MANN (1936) write of partially hepatectomised dogs: "The blood-sugar level may be slightly decreased as compared to the level before operation. However, it is possible to detect this decrease in the level of blood-sugar in the presence of reduced hepatic tissue only by careful control of laboratory conditions, and it would not be noted under ordinary circumstances." And of the glucose tolerance curves compared to curves of normal dogs they state, that they "failed to disclose significant differences".

In totally hepatectomised dogs, on the other hand, hypoglycemia develops quickly. If glucose is injected permanently to such dogs, whereby the blood-sugar is kept normal, administration of glucose shows reduced tolerance. (SOSKIN, ALLWEIS and COHEN, 1934).

This difference may be due to the fact that in the partially hepatectomised dogs sufficient liver tissue has not been removed, the remainder of the liver therefore being able to maintain normal blood-sugar relation.

In rabbits one is able, however, to remove the greater part of the liver, whereupon they become hypoglycemic (MC. MASTER and DRURY, 1929). It is thus possible to examine the glucose

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tolerance at a stage when hepatic insufficiency has been ascertained.

Methods.

The experimental animals were rabbits weighing about 2 kilogram. All operations were aseptic and performed under ether anesthesia. The partial hepatectomies were done partly as indicated by Mc. MASTER and DRURY (1929) partly by a modification of HIMSWORTH's (1938) method for hepatectomy.

By the latter method a preliminary operation is first performed, by which the superior pancreatico-duodenal vein is ligated and a ligature is placed round the portal vein, which decreases the lumen to about 2 mm in diameter. In a couple of weeks collateral circulation has developed. About half of the renal lobe is now extirpated and a ligature is placed round the vessels to the main liver. This ligature, which is of thick cotton yarn, greased with vaseline, is tied but not tightened. The ends of the ligature are brought out of the lateral abdominal walls of the experimental animal and tied on its back. During the days following, glucose-saline is given subcutaneously daily. Four to six days later, when the rabbit has recovered, the ligature is drawn tight. The animal is not anesthetised, reacts only slightly to the procedure and is, a few minutes later, completely natural. In two-three hours the blood-sugar has fallen to subnormal values. The glucose tolerance is now tested.

By post-mortem examination it has been found, that 7—14 per cent of the liver remained in function. Signs of infection or stasis have not been found.

During the last 24 hours before the glucose tolerance test neither glucose-saline nor food was given. 1 gram glucose per kilogram in a 50 per cent solution is injected, the injection is given in the vein of the ear. The blood is taken from the marginal vein of the other ear. The blood-sugar determinations were made with HAGEDORN-NORMAN JENSEN's method. The values stated are the means of duplicate analyses.

Results.

Two rabbits, No. 1 and 2, partially hepatectomised according to the method of Mc. MASTER and DRURY, became hypoglycemic about three hours after operation. At this stage glucose tolerance was examined. In both cases the injected glucose was found to disappear from the blood more slowly than normally (Fig. 1 and Table 1).

Mc. MASTER and DRURY state, that no pronounced dilatation is found of the portal tributaries by the above mentioned method. In order to ensure that the ascertained change in the blood-sugar

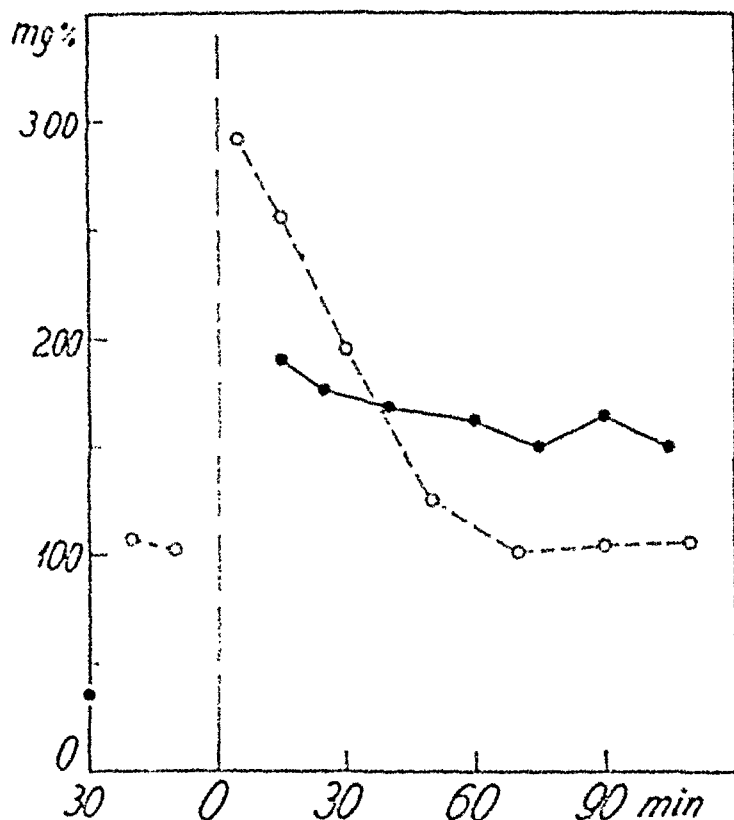


Fig. 1.

Glucose tolerance curve of rabbit No. 1, before (---○---) and after (●—●) partial hepatectomy according to Mc. MASTER and DUNN. At 0 intravenous injection of 1 gram glucose per kilogram.

regulation is not due to stasis, specially in the pancreas, and to exclude a possible influence of narcosis, operation, trauma or fright, the greater part of the liver of four rabbits was shunted out in a manner that excluded these possible mistakes. By a preliminary operation the superior pancreatico-duodenal vein is ligated and the portal vein's lumen decreased. When collateral circulation has then developed, about half of the renal lobe is extirpated and a loose ligature is laid round the vessels to the main liver. Four to six days later the animals have quite recovered. The ligature is now tightened without anaesthesia, whereafter about 90 per cent of the liver is shunted out of the circulation. One, respectively two days before the tightening of the ligature, the glucose tolerance test was performed in two of the rabbits, No. 3 and 4, which showed normal curves (Fig. 2 and

Table 1.

Glucose tolerance of partially hepatectomised rabbits.

Rabbit No. 2 is partially hepatectomised as indicated by Mc. Master and Drury. No. 4, 5 and 6 have been operated upon according to the modified Himsworth's method.

No.	Blood-sugar in mg. pr 100 ml.														Remarks
	Minutes before the glucose injection							Minutes after the glucose injection							
	55	40	35	25	15	10	5	5	15	30	50	70	90	110	
2	59						73 ¹			266		174		160	Injection 4 hours after opr.
4					99	96		334	282	231	179	146	112	100	Lig. placed not tightened
5		54		90 ²		79 ²		393	337	287	285	258	249	245	Liver excluded
5					101		99	313	224	195	137	105	94	104	After pre- liminary opr.
5			61				62	194	210	202		186	161	163	Liver ex- cluded
6							96	332	260	170	116	102	111	112	After pre- liminary opr.
5		47					45	316		188		157	132	124	Liver ex- cluded

¹ Lies with the head on support. No change after glucose injection.

² Lies on the side. Natural after glucose injection.

Table 1). Two to three hours after the tightening of the ligature the blood-sugar of all four rabbits had fallen to subnormal values. Administration of glucose at this stage showed distinctly reduced glucose tolerance (Fig. 2 and Table 1).

Prior to the injection of glucose four of the six experimental animals were slack and lay on their side. They recovered immediately after the injection. Three of them that were not killed after the experiment lived 4, 4½ and 8 hours, respectively, after the glucose injection without signs of hypoglycemia.

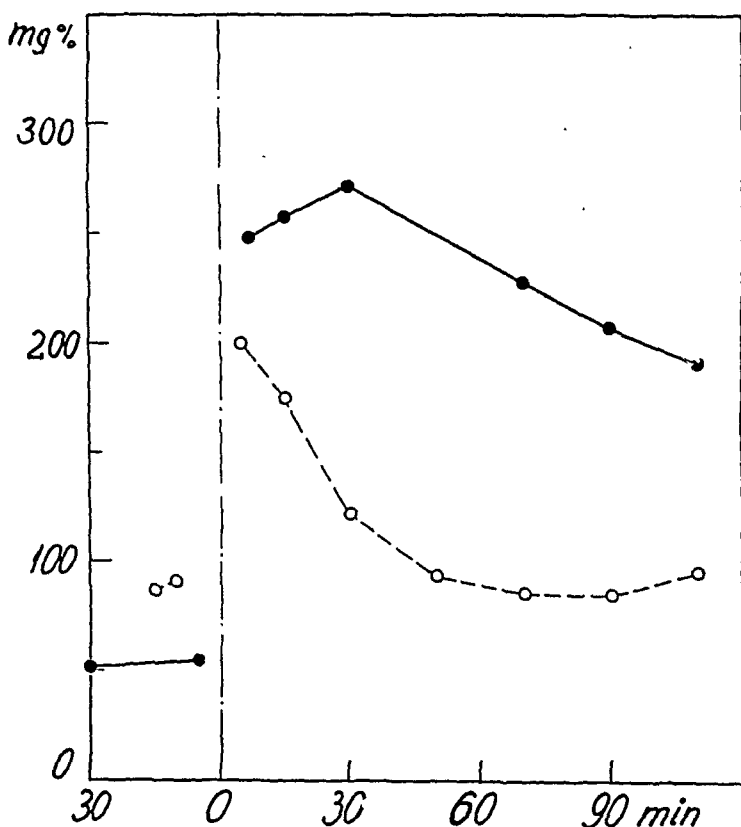


Fig. 2.

Glucose tolerance curve of a rabbit No. 3 after preliminary operation and placing of the loose ligature round the vessels to the main liver (○---○) and after tightening of this ligature (●—●). At 0 intravenous injection of 1 gram glucose per kilogram.

Discussion.

The experiments show that when, after partial hepatectomy, rabbits have become hypoglycemic, intravenously injected glucose disappeared more slowly than normally from the blood.

The control experiments show that this is not due to stasis in the portal circulation, just as it has been excluded, that anesthesia, operation, trauma, infection or fright could have been the cause of this change. Consequently it must be ascribed to reduction of the liver tissue.

BOLLMAN and MANN found no distinct changes in the blood-sugar regulation of dogs after removal of 80 per cent of the liver. MC. MASTER and DRURY's rabbits became hypoglycemic, when

more than 80 per cent of the liver was removed. In the experiments published in this paper hypoglycemia and reduced glucose tolerance were demonstrated after removal of about 90 per cent of the tissue. Consequently, the liver seems able to maintain normal blood-sugar regulation even when so small a part as twenty per cent remains in function.

Summary.

Hypoglycemia combined with reduced glucose tolerance has been demonstrated in partially hepatectomised rabbits.

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A Micromethod for the Determination of Alanine in Proteins.

By

STIG E. G. ÅQVIST.

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The methods available for the determination of alanine are in some respects less satisfactory.

In one of the best known methods, that of FÜRT, SCHOLL and HERRMANN (1932) the alanine is converted into lactic acid by means of nitrous acid and the lactic acid is oxidized to acetaldehyde, which is determined titrimetrically with iodine. (FRIEDEMANN, COTONIO and SCHAFFER 1927). More recently BLOCK, BOLLING and WEBB (1940) determined the lactic acid thus produced by oxidising it with periodic acid to acetaldehyde which in turn is determined colorimetrically with p-hydroxybiphenyl. The first method requires rather large amounts of proteins, and some amino acids which interfere with the reaction must be removed prior to the analysis. In the second method threonine, which yields acetaldehyde under the same conditions, is estimated simultaneously.

VIRTANEN, LAINE and TOIVONEN (1940), and ALEXANDER and SELIGMAN (1945), determined alanine by means of ninhydrin (triketo-hydrindene-hydrate), which reacts with alanine to give acetaldehyde. The acetaldehyde is distilled off and estimated colorimetrically with p-hydroxybiphenyl.

The present method is based upon the conversion of alanine to lactic acid by nitrous acid and the excess of nitrous acid is then destroyed by hydrazine hydrochloride. The lactic acid is converted into acetaldehyde by hot concentrated sulphuric acid

and the acetaldehyde is determined with p-hydroxybiphenyl according to KOENEMANN's modification of the Miller and Muntz method (1940).

Reagents.

1. Glacial acetic acid p. a.
2. Sodium nitrite p. a. To 2.5 gm is added distilled water to make a volume of 100 ml. A fresh solution is made every day.
3. Hydrazine hydrochloride p. a. 7.5 gm in 100 ml of distilled water.
4. Sulphuric acid p. a. sp. g. 1.84 (Kahlbaum).
5. p-hydroxybiphenyl, purified according to MILLER and MUNTZ (1938). 225 mg of the recrystallized preparation is dissolved in 10 ml of 0.18 M sodium hydroxide. If crystals are formed on standing they should be redissolved by heating the solution before use. The solution has been kept in a dark bottle for over a month without evidence of deterioration.
6. Alanine standard. A stock solution is prepared by dissolving 10 mg of alanine in 100 ml of distilled water.

Procedure.

The protein to be analyzed is hydrolyzed with hydrochloric acid in the usual way, and excess acid is removed by repeated evaporations in vacuo. For reasons discussed below the basic, neutral and acidic amino acids of the protein hydrolysate are separated by means of (THEORELL and ÅKESSON 1942) electro-dialysis.

1 ml of the neutral fraction containing 50 γ —200 γ of alanine is placed in a round-bottomed dish, and 0.5 ml of glacial acetic acid is added. The dish is placed on a boiling water-bath. In the course of five minutes, 1.5 ml of the sodium nitrite solution is added drop by drop, while the dish is rocked gently. The sample is heated for another five minutes. Water is added during the heating so that the volume becomes about 2.5 ml. Now 1.5 ml of hydrazine hydrochloride solution is added and the sample is left to cool off at room temperature, and then diluted to exactly 5 ml.

Samples of 0.2 ml are transferred to test-tubes $5/8 \times 5$ inches equipped with ground glass stoppers, which are placed in an ice-bath. 1.5 ml of conc. sulphuric acid is added drop by drop, the test-tubes being gently shaken. The test-tubes are sealed and heated in a boiling water-bath for 5 minutes, and then cooled in an ice-water bath for at least 10 minutes. The glass stoppers are taken off, and 0.02 ml of p-hydroxybiphenyl reagents is ad-

ded from a micropipette. The contents are mixed carefully by shaking the test-tubes.

The blue-violet colour is developed during 1 hour at room temperature or 30 minutes at 30° C. Next the samples are boiled for 30—40 seconds, and are then read in a step-photometer, using filter S₇, and 0.5 cm cuvettes.

Discussion.

When the colour reaction with p-hydroxybiphenyl was applied directly to the lactic acid formed after deamination of the alanine it turned out that the excess of nitrous acid interfered with the colour reaction giving rise to an intense green colour. Consequently the nitrous acid had to be eliminated, and for this purpose the addition of hydrazine hydrochloride was found to be suitable.

In order to increase the sensitivity of the colour reaction BARKER and SUMMERSON (1941) recommended the addition of copper. According to the author's experience this addition diminishes the constancy of the method and has been omitted. A protein to be analyzed for alanine, however, possibly contains sufficient amounts of copper to interfere with the estimation. Then it is recommended as a rule first to submit the hydrolysate to electro-dialysis and to determine alanine in the neutral amino acid fraction which is free from metal ions. The author has made use of the convenient electro-dialysis procedure of THEORELL and ÅKESSON (1942).

The following amino acids have been found not to produce any colour in amounts twenty times greater than that of alanine: Arginine, histidine, lysine, glycine, serine, threonine, tyrosine, tryptophane, phenylalanine, cystine, cysteine, methionine, proline, hydroxyproline, leucine, isoleucine, valine, aspartic acid, and glutamic acid.

Summary.

A method is described for the colorimetric determination of alanine in protein hydrolysates. Alanine is converted into lactic acid by means of nitrous acid. The lactic acid is oxidized to acetaldehyde by warm concentrated sulphuric acid, and the acetaldehyde is estimated by its colour reaction with p-hydroxybiphenyl. The colour is read in a Pulfrich photometer using filter S₇. By this method, 0.05—0.2 mg of alanine can be estimated fairly accurately.

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Stalagmometric Determination of Normal Serum Esterase and Pancreatic Lipase in Serum.

By

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In two recent works the possibilities of differentiating between the normal serum esterase and the pancreatic lipase in serum were elucidated (LAGERLÖF, 1944 & 1945). In this work the definite methods are reported.

The esterase activity of normal serum almost exclusively depends upon the activity of the normal serum esterase. This esterase is inactivated to about 95 per cent by addition of 1 ml. of a 2 per cent solution of atoxyl per ml. of serum. It is not activated by calcium oleate.

Pancreatic lipase in serum is completely resistant to amounts of atoxyl which cause submaximal inhibition of normal serum esterase. Its activity is increased at least fiftyfold by the addition of calcium oleate.

Therefore it may be determined separately from the serum esterase if the latter is inhibited submaximally with atoxyl and the pancreatic lipase is activated with calcium oleate.

A small error may be caused by small amounts of serum esterase which is not inactivated by atoxyl. This error is corrected by subtracting the activity which is found when calcium oleate is not added.

Methods.

Solutions required:

1. Saturated tributyrin solution (LAGERLÖF, 1945).
2. Buffer Solutions: 1 part of a 4 molar solution of NH_4OH + 4 parts of a 4 molar solution of NH_4Cl . (pH about 8.8).
3. 0.25 per cent CaCl_2 solution.
4. 0.5 per cent sodium oleate solution.
5. 2 per cent atoxyl solution. Should be prepared with boiled water. Keeps about 14 days in a dark refrigerator.

Procedure. The serum is diluted in the proportions 1 to 2 or 1 to 4 with distilled water. 1 ml. of the dilution is mixed with 1 ml. of atoxyl solution in a small test tube and then remains 25 to 40 minutes at room temperature. The serum is then "poisoned". The tributyrin is warmed to room temperature. For every esterase determination 50 ml. are pipetted over to 100 ml. Erlenmeyer flasks, which are then placed in a water bath of 30 C. Ten to sixty minutes later 1 ml. of buffer, 1 ml. of sodium oleate and 1 ml. of CaCl_2 in the order given are added. At a time noted to the second the poisoned serum is added and thoroughly mixed with the substrate. The drop counting is then begun as soon as possible, generally 90 seconds later. The number of drops at the beginning of the hydrolysis is extrapolated from the value thus obtained.

Further drop countings are done during the course of the hydrolysis, 5 to 60 minutes after the adding of the serum. Drop differences of 5 to 15 drops are desired when stalagmometers with a drop number for water of 60 are used. The drop counting after t minutes of digestion is begun at $t - \frac{r}{2}$ seconds, the running out time being r seconds.

From the drop difference and the time of digestion the reaction constant C is determined according to the formula $C = \frac{1}{t} \log \frac{91}{x}$ where t represents the digestion time in minutes and x the concentration of the tributyrin at the time t , measured in per cent of saturation. In routine work it is obtained from the empirical table 3. The reaction constant times 1 000 is used to express the esterase activity.

At the same time as this determination the esterase activity is determined by exchange of the calcium chloride and sodium oleate with distilled water. The esterase activity obtained in this determination is subtracted from the activity obtained with activation. The difference represents the activity of the pancreatic lipase.

The normal serum esterase is determined in the same way without the addition of atoxyl, calcium chloride and sodium oleate.

Practical Notes on the Determination of the Esterase Units per Ml. Drop numbers obtained with different stalagmometers according to Traube may be compared directly, if the drop rate is very slow. If the drop rate increases the drop number will decrease at least in the beginning (table 1). This little affects the drop differences which occur

Table 1.

Drop Numbers of Substrates Containig Different Concentrations of Tributyrin. (Expressed in per cent saturation.)

20 C. Addition of inactivated serum, buffer and calcium oleate in the amounts used. Drop number for water of stalagmometer A 53.5, B 23.4, C 60.2 and D 36.6. Correction for the different drop numbers. All figures express the mean of triple determinations.

Stalagmometer	91 %	68.75 %	45.5 %	22.75 %	0 %
A. Drop rate for water 13/min.	157.3	152.6	146.3	138.6	120.1
B. Drop rate for water 16.5/min.	154.5	149.2	144.7	136.3	117.5
C. ¹ Drop rate for water 27/min.	149.2	144.2	138.7	130.5	110.7
C. Drop rate for water 38.1/min.	145.7	141.4	135.5	127	105.2
D. ¹ Drop rate for water 35.4/min.	142.4	137.6	132.1	124.4	103.8
D. Drop rate for water 54.8/min.	149.7	144.3	137.2	127.8	106.6

Table 2.

Relation between Drop Difference and Concentration of Esterase in Units per ml.

Normal stalagmometer. Serum dilution $\frac{1}{4}$. Time of digestion 30 min./30 C.

Drop difference	0	2	4	6	8	Drop difference	0	2	4	6	8
0.		0.2	0.4	0.6	0.7	13.	11.2	11.4	11.6	11.7	11.9
1.	0.9	1.1	1.2	1.4	1.5	14.	12.1	12.3	12.5	12.7	12.9
2	1.7	1.9	2.1	2.2	2.4	15.	13.1	13.2	13.4	13.6	13.8
3.	2.6	2.7	2.9	3.1	3.2	16.	14.0	14.2	14.4	14.6	14.8
4.	3.4	3.6	3.7	3.9	4.1	17.	15.0	15.1	15.3	15.5	15.7
5.	4.3	4.4	4.6	4.7	4.9	18.	15.9	16.1	16.3	16.5	16.7
6.	5.1	5.3	5.4	5.6	5.7	19.	16.9	17.0	17.2	17.4	17.6
7.	5.9	6.1	6.3	6.4	6.6	20.	17.8	18.0	18.2	18.4	18.6
8.	6.7	6.9	7.1	7.2	7.4	21.	18.8	19.0	19.1	19.3	19.5
9.	7.6	7.7	7.9	8.1	8.3	22.	19.7	19.9	20.1	20.3	20.5
10.	8.4	8.6	8.8	9.0	9.2	23.	20.7	20.9	21.1	21.3	21.5
11.	9.3	9.5	9.7	9.9	10.1	24.	21.7	21.9	22.1	22.3	22.5
12.	10.3	10.5	10.7	10.9	11.1	25.	22.7	22.9	23.1	23.3	23.5

during an esterase determination, if the drop rate for water is less than 40 drops per minute, and consequently little affects the reaction constant, if this is calculated from the drop differences according to table 2.

The use of table 2 is best understood by the following example: Assume that the serum dilution is $\frac{1}{4}$, the digestion time 15 minutes, the drop number for water of the stalagmometer 50 drops and the drop

¹ The stalagmometer attached to a capillary, to diminish the drop rate.

difference 5.0 drops. The drop difference for a "normal stalagmometer" which has a drop number for water of 100 is then $\frac{100 \times 5.0}{50} = 10$.

In the table 10.0 drops corresponds to 8.4 units. The number of units per ml. will then be $\frac{4 \times 30 \times 8.4}{2 \times 15} = 36.0$

The Standard Deviation of the Esterase Methods. The total serum esterase (SE) and the atoxyl resistant serum esterase determined without calcium oleate (AE) as a rule were determined in single tests. Two drop countings were made at the beginning and two at the end of the digestion. The atoxyl resistant serum esterase determined with activation (AE_n) was usually determined in two different tests. As a rule two drop countings were done before and one at the end of digestion in the first test and in the second test one counting before and two at the end. The reaction constant was calculated for each test.

The standard deviation of the esterase values expressed in units is dependent not only upon the accuracy of the drop counting, but also upon other factors, whose influence cannot be dealt with statistically. Such factors are the exactitude and shape of the standard curve. Nevertheless, a statistical determination of the standard error of the methods used may be of value.

The standard deviations of the determinations of SE, AE and AE_n were calculated from available double tests with the use of the formula

$\sigma = \sqrt{\frac{d^2}{2n}}$ (table 3). While AE_n was always determined in double tests, the standard deviation of the double tests is $\frac{\sigma}{\sqrt{2}}$. The standard

Table 3.
Standard Deviations of the Esterase Methods.

Method	Esterase values between	Number of double determinations	Mean	Standard deviation, σ
Serum esterase .	9.1 —31.2 units/ml	16	23.9 \pm 1.6	1.49 \pm 0.26
	32.7 —78.3 "	16	42.1 \pm 3.1	2.05 \pm 0.36
	9.1 —78.3 "	32	33.0 \pm 2.4	1.79 \pm 0.22
Atoxyl resistant esterase determined without Na-oleate	0.90— 3.12 "	20	2.02 \pm 0.13	0.479 \pm 0.076
Atoxyl resistant esterase determined with Na-oleate	1.26— 4.05 "	29	2.31 \pm 0.14	0.439 \pm 0.058
	4.27—18.2 "	21	10.77 \pm 0.30	0.94 \pm 0.15

deviation of the determination of pancreatic lipase (PL) i. e. the difference between AE_a and AE , is calculated with the use of the formula $\sigma_{PL} = \sqrt{\sigma AE_a^2 + \sigma AE^2}$, in which σAE_a is the standard deviation of AE_a and σAE of the determination of AE_x . For low, i. e. normal values, it amounts to 0.57 units per ml.

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The Influence of Morphine Hydrochloride on the Pancreatic and Biliary Secretion in Man.

By

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Though morphine is widely used in the relief of pain in acute pancreatic disease, its action on pancreatic secretion in man has not been investigated, except in a preliminary work of the author (LAGERLÖF, 1944). Animal experiments show that it decreases the volume of pancreatic secretion (BABKIN, 1928), but there is no information regarding the pancreatic enzymes.

In this work the effect of morphine was studied with a method previously used for investigation of the effect of insulin, pilocarpine, atropine and adrenalin on the pancreatic secretion in man (LAGERLÖF 1942). The duodenal secretion after secretin is compared with the secretion after injections of secretin and morphine intravenously. Three experiments were done, all in cholecystectomized patients. Table 1 shows one of them.

The volume of duodenal juice was considerably diminished by the injection of morphine in spite of the fact that the duodenal secretion in 2 of the cases was high when the second secretin injection was done. In two of the cases the decrease was significant.

The bicarbonate concentration of the duodenal juice was little affected. The total amount of bicarbonate per unit of time was lowered in all cases, significantly in two.

In spite of the lowered rate of secretion the concentration of amylase decreased to between a fifth and a tenth of the concentration in the juice secreted after secretin alone. The same lowering

Table 1.

The Effect of Morphine Hydrochloride on the Duodenal Secretion after Secretin.

Ex- peri- ment	Frac- tion	Time min.	Vol- ume ml	Bicarbonate		Amylase		Bilirubine		Remarks
				M. Eq. per l.	Eq.	units per ml	units	index	index x vol- ume	
1 a	0	20	28					126	3530	Secretin, 140 clinic- al units i.v.
	1	10	59	27	1.59	4.42	276	68	4010	
	2	10	36					35	1260	
	3	20	63					33	2070	
	4	20	38					36	1360	Secretin, 140 clinic- al units i.v. Morphine HCl, 15 mg i.v. Severe pain at onset.
	2-4	50	137	86	11.8	2.10	288	36	4690	
	5	20	40	59	2.36	1.39	55.7	34	1360	
	6	20	66	53	3.50	1.29	85.1	40	2120	
	7	5	33	72	8.9	2.30	82.0	38	1250	
1 b	8	5	21	77	1.68	1.30	27.3	34	714	Pain at onset.
	9	5	15	77	1.16	0.10	1.3	1.3	26	
	10	5	12	82	0.88	0.098	1.2	0.3	6	
	11	5	7					0	0	Pain at onset.
	12	5	4.5	92	1.06	0.093	1.1	0.3	3	
	13	5	11					22	242	
	14	5	8	84	16.0	0.26	3.3	30	240	Pain at onset.
	15	5	17							
	16	5	1	75	1.33	0.33	6.3	37	629	
	17	5	11					47	517	Pain at onset.
	18	5	16	79	2.13	0.58	15.7	54	864	
	9-18	50	103		8.11		29.3		2527	
	19	5	17					36	612	Pain almost ceased
	20	5	5	73	10.1	0.77	16.9	32	160	
	21	5	3.5					30	105	
	22	5	10					45	450	Pain almost ceased
	23	5	16	74	2.85	0.96	37.0	65	1040	
	24	5	9					68	612	

was found concerning the trypsin. As the amount of enzymes secreted per unit of time is a function of volume and concentration they decreased to a somewhat higher degree.

The bilirubin concentration was lowered in all cases and in two cases the juice was colorless during 15 and 20 minutes respectively. The total amount of bilirubin was decreased from 2 to several times.

Two of the patients developed attacks of pain 5 and 30 minutes respectively after the end of the morphine injection. The same occurred in another case investigated with a somewhat different technique of fractioning the juice. The decrease of volume, enzyme

and bilirubin concentration appeared between 5 and 30 minutes before the pains.

Discussion.

In secretin tests on normal persons the bile color of the duodenal juice disappears 5 to 10 minutes after the secretin injection and remains so for 10 to 60 minutes, in spite of increased biliary secretion. The bile is evidently diverted into the gall bladder. This is probably not accomplished by contraction of the duodenal musculature or the lower part of Oddi's sphincter, as the pancreatic secretion continues at a high rate. It must then be caused by contraction of the upper part of Oddi's sphincter, sphincter choledochus (BOYDEN). Alkalizing of the duodenal content may be the cause, as the juice remains colored in secretin tests on patients with failure of the pancreatic secretion.

In cholecystectomized patients and in patients with a non-functioning gall bladder the bile flows continuously during the secretin test. Evidently the increased tonus of sphincter choledochus is not sufficient to retain the bile against the secretion pressure when this is not counterbalanced by a functioning gall bladder.

In two of the secretin experiments here reported on cholecystectomized patients the flow of bile ceased for 15—30 minutes. The duodenal juice which was simultaneously secreted contained bicarbonate trypsin and amylase of far higher concentrations than the duodenal juice in complete obstruction of the pancreatic ducts. Therefore some pancreatic secretion must have remained. As contraction of the duodenal musculature should hinder the flow both through the duct of Wirsung and the duct of Santorini, the cause of the ceased bile flow seems to be contraction of Oddi's sphincter.

The lowered amounts of bicarbonate after the morphine injections indicate decreased delivery of pancreatic juice to the duodenum. That this at least in part is due to contraction of Oddi's sphincter and resulting pancreatic stasis is borne out by a simultaneous rise of the pancreatic enzymes in the serum shown in other experiments (LAGERLÖF, 1945).

The amounts of amylase and trypsin decrease several times more than the amounts of bicarbonate. The decrease therefore mainly depends on decrease of the production of pancreatic enzymes.

Summary.

Morphine decreases the delivery of bile and pancreatic juice to the duodenum. This is due, partly at least, to contraction of Oddi's sphincter. Morphine depresses the production of pancreatic enzymes.

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Serum Phosphatase in Rats with Obstructive Jaundice.

By

JÖRGEN B. DALGAARD.

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Since ROBERTS in 1930 demonstrated increased serum phosphatase in two cases of obstructive jaundice, such increase has been shown experimentally by numerous investigators in dogs (BODANSKY and JAFFE 1934; FREEMAN, CHEN and IVY 1938; SCHIFFMANN and WINKELMAN 1939; GUTMAN, HOGG and OLSON 1940, CARR and FOOTE 1944; GAD 1945; WACHSTEIN and ZAK 1946) and in rabbits (JALLING 1945; JALLING, LAUERSEN and VOLQUARTZ 1945; HOFFMEYER, JALLING and SCHÖNHEYDER 1946; IBSEN 1945; a. o.). Clinically, the estimation of serum phosphatase, or more correctly, the phosphomonoesterase with pH optimum about 9.1, is now in common use all over the world as a means of differentiating obstructive jaundice (with increased serum phosphatase) from infective jaundice (with slightly or not increased phosphatase), (ROBERTS 1933; AUSTONI and COGGI 1934; HARTMAN and SCHELLING 1934; FLOOD, GUTMAN and GUTMAN 1937; CANTAROW and NELSON 1937; SHARNOFF, LISA and RIEDEL 1942), most often in combination with other liver function tests (LEHMANN 1941; BUCH 1942).

Nevertheless we do not exactly know the *reason* for this phosphatase increase. The different theories will not be discussed here. But pathophysiologic problems are often elucidated by comparing animals showing different responses to special operations. With regard to the above mentioned statements, which all conform,

it is noteworthy that according to a few authors two animals should react in quite a different way following bile duct ligation. CANTAROW, STEWART and MCCOOL 1936, thus state that the cat does not display phosphatase increase, which finding is later on sustained by THANNHAUSER, *et alii* (1937). This question will be discussed in another paper. Of more interest here, however, is the investigation carried out in 1942 by WEIL and RUSSEL, who communicate that in the rat they find no increase in serum phosphatase following ligation of the common bile duct, but on the contrary a distinct drop.

The author has revised and extended these experiments. The results obtained do not agree with the findings of WEIL and RUSSEL. As these authors are often quoted and their results used as a basis for considerations of principles (MOOG, 1946) and as it seems that no others have performed the experiments on rats, their work should be briefly summarized here:

Weil and Russel's Previous Findings in Rats.

WEIL and RUSSEL used normal albino rats. The serum phosphatase was estimated by means of an ultra-micro method, using Mg^{++} -activated Na- β -glycerophosphate in the substrate, which was buffered to pH 9 by veronal-HCl. A modified BODANSKY method (1931) was thus utilized, which in its principle resembles the one used here. Direct comparison between the results is thus possible.

The operations, performed in amytal-ether narcosis, consisted in simple ligation of the common bile duct. Icterus was noticed during the first day:

"The plasma phosphatase activity dropped markedly as early as a day after the operation. The phosphatase level remained low over a period of 6 to 8 days, after which the enzyme activity increased rapidly and rose above the post-operative level and then gradually returned to normal. While the sudden drop in plasma phosphatase activity resulted from obstruction of the bile ducts, the rise which followed was associated with the functioning of new bile ducts, as indicated by the disappearance of jaundice symptoms and by postmortem examinations".

If 3 % of heat inactivated oxbile was added to the food, no drop in phosphatase was noticed.

On p. 9 the author discusses the results of WEIL and RUSSEL.

Here need only be stressed that the rats were fed with purina dog chow during the whole experiment.

In another work from 1940 the same authors had shown that the serum phosphatase activity in rats drops during fasting, reaching a minimum in about 16 hours. (According to the findings of the present author the minimum was not reached until after 24—48 hours fasting.) WEIL and RUSSEL stated that only the alcohol-ether soluble extract from the food can augment the phosphatase level and through extensive experiments it was demonstrated that only fatty acids of a certain molecular size ($> C_{11}$) containing double bonds and free carboxyl-groups, but neither protein nor carbohydrates are able to increase the serum phosphatase.

This work was continued by GOULD (1944) who showed that by feeding rats with excessive doses of fat it is possible to augment the serum phosphatase activity to a level considerably higher than the normal.

Experimental.

The rats: Adult albino rats of the Wistar type with a weight of more than 180 g. are used. This type has been bred in repeatedly during several years and is as uniform as possible.

Anatomically: Rats have no gall-bladders. A hepatic duct is given off from each of the separate liver lobes and these ducts soon unite into a single choledochus, which in its last part passes in near connection to the pancreas, from which small accessory pancreatic ducts are taken up (comp. OPPEL 1900).

In order to investigate, whether new bile ducts are developed after the operation methyl-green was injected into a closed duodenal fragment in which the common bile duct opened, after which the hepatoduodenal ligament was microscopied. In no case new bile ducts were seen, as opposed to the finding claimed by WEIL and RUSSEL.

The operations are performed in ether narcosis, which must be administered with caution, in order to avoid killing the rats. A 2 cm. cross section is cut through the skin and medially through the muscles and peritoneum. The pylorus is drawn forth from the depth and 15—20 mm. beneath this the choledochus opens into the duodenum. The duct is cautiously dissected free at a place 1 cm. from its termination. At a lower level one might destroy the small pancreatic ducts, and at a higher level the hepatic duct from the lower posterior liver lobe might escape the ligature. The isolated choledochus is squeezed with two Cushing's arterial clips and divided between them, thus ensuring that the animal will develop cholascus and die, if the chole-

dochus is insufficiently closed. Otherwise this would compromise the results. A tip of omentum is placed over the field of operation, saturation in two or three layers, with agraphs in the skin, as the animals sometimes bite the sutures. During and after the operation the animals are placed on a hot plate at 35°.

Following the operations the animals develop icterus in the course of one day which is seen by yellowing of the ears, the paws and by increased serum icterus index, which is normally less than 10 (Meulengracht).

Controls: In each group of 4—8 animals at least one is sham-operated, i. e. the choledochus is isolated, but not ligated or cut. Some animals are only ether anaesthetized in order to ensure against a possible ether-effect. Phosphatase estimations before, during and after ether anaesthesia, however, show no deviations.

Blood withdrawing: Blood is obtained with or without suction from the tail which must not dip into the blood. Neither must too high a vacuum be used, as this promotes hemolysis, and hemolysed blood is inapplicable, as the blood corpuscles contain much less (alkaline) phosphatase than the serum, the phosphatase of which is thus diluted through hemolysis.

Experimental:

Phosphatase in a pure serum: 135.

In the same serum after (freezing)-hemolysis: 109.

The serum phosphatase must be determined soon after the withdrawal of the blood (within 1 hour here) because of the paradoxical increase during preservation of serum even in a refrigerator, which was first noticed by BODANSKY (human serum?) later on noticed by JALLING (rabbit serum) and now also seen by the author in rat serum.

The phosphatase determinations are carried out according to LUNDSTEEN-VERMEHREN (1936) (a micromodification of BODANSKY's method), but instead of blood, serum is used. Thus hematocrit determinations are avoided. Because of the considerable amount of phosphatase in rat serum only two hours of hydrolysis is used. This also involves optimal conditions for the enzyme, as seen in fig. 1, which shows a direct proportionality between time of hydrolysis and phosphorus liberated within 4—5 hours.

One unit of phosphatase is defined as the amount of enzyme liberating 1 mg. of phosphorus per 100 cc. serum during the circumstances stated (2 hours of hydrolysis at $37.4^{\circ} \pm 0.1^{\circ}$, Mg^{++} -activated

Na- β -glycerophosphate as the substrate, pH 8.87, buffered with $\text{NH}_4\text{OH}-\text{NH}_4\text{Cl}$).

The estimations of the phosphatase values are made in the following way:

The reading (extinction) on the scale of the Pulfrich photometer is termed e . The reading of a known standard-solution, containing 5 mg.

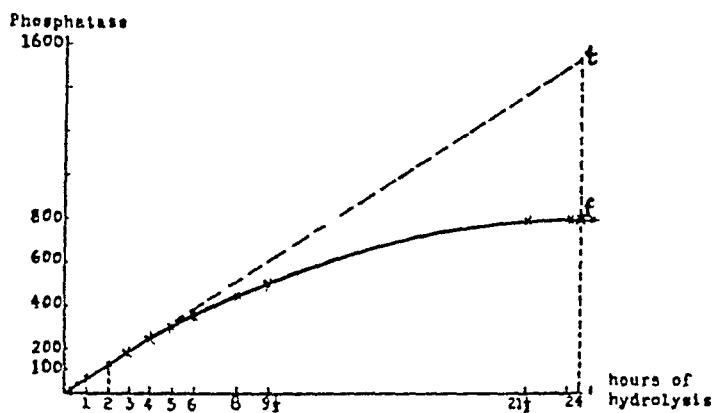


Fig. 1. Time curve of hydrolysis with constant enzyme amount.

Note that within 4—5 hours there is complete proportionality between hydrolysis-time and amount of phosphorus liberated (phosphatase). The 24 hour value found (e), however, is much lower than the theoretic value (t) estimated according to continued proportionality.

This curve is a mean of 8 experiments.

per cent of phosphorus, or in the 400 cmm. used 0.001 mg. of P, is termed \bar{E} .

The amount of P liberated by the sample investigated is then:

$\frac{e}{\bar{E}} \cdot 0.001$. The amount of P liberated by 100 cc. of serum is

$$\frac{e}{\bar{E}} \cdot 0.001 \cdot 18\,375$$

(See formula by LUNDSTEEN-VERMEHREN, p. 163, in which $pl = 1$, as serum is used here). In practice is found $\bar{E} = 0.20$ which put into the formula makes the amount of P liberated by 100 cc. of serum, or the phosphatase value $= e \cdot 92$.

Statistic Treatment of the Material.

When observations are made on rats fed on a mixed diet, average values of serum phosphatase have little significance as the dispersion is very large. When 48 hour starvation values are used, however, statistic treatment is reasonable. In 26 rats 35 fasting values have been

determined. The mean is 55.6 units of phosphatase with a coefficient of variation of 18.7 per cent.

The differences between the double determinations give a measure on the accuracy of the method. This is, however, very different in different measurement groups. Therefore the results are arranged in groups and the accuracy estimated for each group alone. (The figure (e) indicates the readings on the scale of the Pulfrich photometer.)

- Group 1: $e < 1$ (phosphatase < 92). In this group, which includes all 48-hour starvation values, the accuracy is highest. In 189 double determinations, that means 378 analyses σ is found 2.234, or the standard error of the mean of double determinations 0.0158 (e) or 1.5 phosphatase units.
- Group 2: $1 < e < 1.5$ (phosphatase between 92 and 138). In 156 analyses $\sigma = 4.292$, standard error 0.0335 (e) or 3.1 phosphatase units.
- Group 3: $e > 1.5$ (phosphatase > 138) 176 analyses. $\sigma = 10.55$, standard error 0.0746 (e) or 6.9 phosphatase units.

It is thus seen that the accuracy is rather satisfactory (standard error less than 1.5 phosphatase units), when phosphatase values less than 92 are concerned. It is also noticed, which could be expected, that the accuracy decreases as the phosphatase increases. Therefore the samples are diluted before reading the Pulfrich photometer in all cases where, according to the darkness of the coloured sample, a value of $e > 1$ is to be expected.

Experimental Results.

The first, misleading experiments: At first the author did not realize the importance of a long continued fasting in order to obtain a minimal and constant serumphosphatase level as starting point for the experiments. Following the statements of WEIL & RUSSEL (1940) it was considered sufficient when the animals had been starved 10—16 hours before the operation. The first results were like those of W. & R. 7 operated rats out of 12 showed a drop in serum phosphatase from the 1st to the 3rd day. After the 4th day the 5 animals still alive showed the beginning of an increase which, however, only in the two animals who survived longest surpassed the starting level (on the 6th and 9th day). In one animal the phosphatase increased steadily up to 1950, at the last estimation on the 35th day, while the other animal, after a maximum on the 15th day showed a slight drop on the 23rd day, which, however, is possibly only premortal.

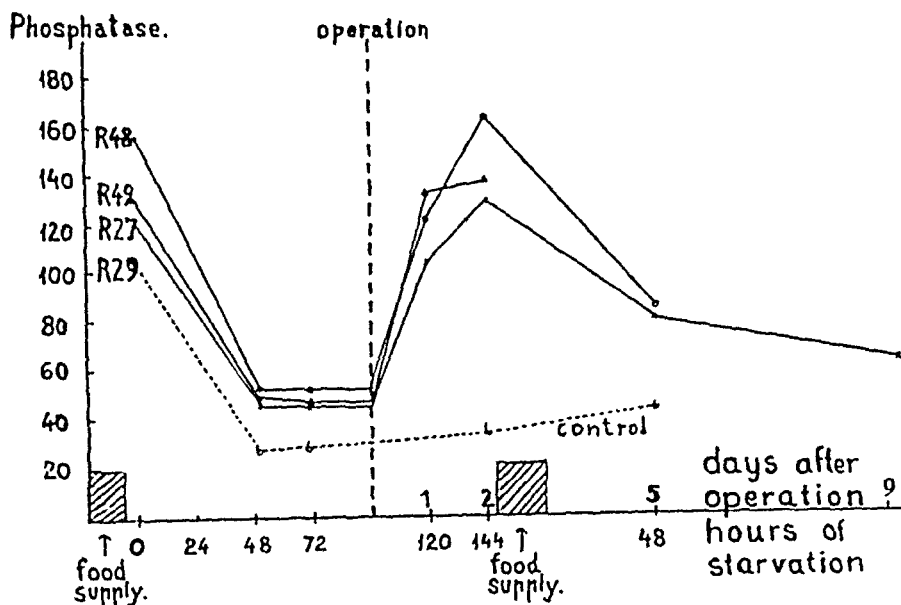


Fig. 2. During 48 hours of starvation a marked drop in serum phosphatase to minimal and constant levels is obtained. Following ligation of the common bile duct an immediate and conspicuous increase is noticed, as opposed to the un-operated control.

In these early experiments, however, 3 other rats showed a distinct increase in serum phosphatase already the day after the operation. In one of these it later on dropped towards the starting level just before death on the 9th day, while the two others died resp. on the 1st and the 4th day.

These early experiments thus yielded different and uncertain results.

The following, true experiments: The author found that the serum phosphatase in rats often becomes minimal and constant only after 48 hours fast. Further, the phosphatase level proved extremely dependent on the fat metabolism (comp. W. & R.). When this had become clear, the experiments were repeated with series of rats, starved 48 hours or more previous to the operation.

In fig. 2 an especially significant series is shown, in which the animals were starved not less than 96 hours previous to the operation. First the very considerable drop in serum phosphatase during the starvation period is noticed. After 48 hours starvation the phosphatase level is very low and, which is of special interest, quite constant. This is demonstrated here through another 24

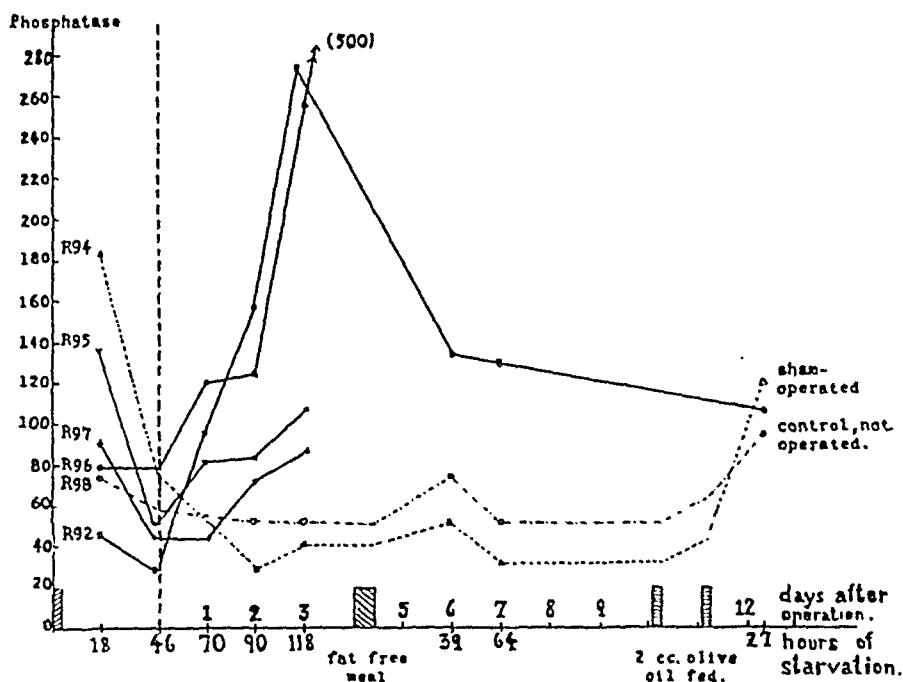


Fig. 3. A marked increase in serum phosphatase follows bile duct ligation in 4 rats whose phosphatase levels have been reduced previous to the operation through 48 hours of starvation.

The sham-operated and the control show fairly constant low levels that are only slightly elevated by supplying a fatfree meal, but remarkably so by feeding olive oil, which, however, does not seem to influence the operated animal (R 92).

Note that the upper part of the ordinate is much reduced in order to save space.

hours starvation period, which does not reduce the level any more. Further it is noticed that all the animals which belonged to the same litter have quite similar values.

The day after the operation the phosphatase has increased to three times the normal starving level and the second day the values are still higher. Then the animals must eat but after a further 48 hours starvation, that means, on the 5th day the phosphatase level is still about twice the preoperative level although lower than on the 3rd day. The single estimation on the 9th day indicates that the slow drop continues. The other animals died resp. on the 5th and 9th day.

The maximal level need not be reached by the 3rd day. This has been hinted at in the earlier experiments and is also seen in fig. 3. All of the operated animals in this series show phosphatase increases, which in two animals reach very high degrees already on the 3rd day. While in one animal the increase

continues steadily, the other one displays a slight drop, visible on the 6th day. R 97 and 96 die resp. on the 4th and the 8th day, while R 95 and 92 are killed resp. on the 3rd and 18th day.

Note the control-animal and the sham-operated, which show constant and low phosphatase levels until they later show a moderate increase after feeding the standard diet without fat and finally a considerable increase after feeding olive oil, which does not influence the operated R 92. (See further in a coming paper.)

It should be noted that rats dying early after the operation most often have a rather low phosphatase level. Even animals which earlier showed an increase have mostly low figures if examined immediately before they succumb. Because of that, one can not lay stress upon the *slight degree* of phosphatase increase displayed by R 95 and R 97 in fig. 3. In these cases rats with complications (peritonitis) after the operation are concerned. Just these cases are shown here in the figures in order to demonstrate another possible source of error in experiments of this sort. It should be added here, that the large majority of rats surviving the operation more than two days (in all 27 animals) displayed a distinct increase in phosphatase.

Discussion.

In fig. 2 the curves are presented in a way allowing comparison with the curves of WEIL and RUSSEL (1942). It is conspicuous, how much the two sets of curves are alike, with respect to the common form. In the authors' curves, fig. 2, however, the drop in serum phosphatase occurs *before* the operation, brought about, as mentioned, simply through starving the rats 48 hours. In WEIL and RUSSEL's experiments, on the contrary, the rats have been fed during the whole experiment. But have they eaten the food? According to the author's observations newly operated animals do not eat significantly, they only drink water. WEIL and RUSSEL, who themselves have worked on the influence of the food, especially its fat fraction, on the serum phosphatase, ascribe the decrease they noticed after the operation to an insufficient fat resorption. The present author in so far is in conformity with this opinion but considers the faulty fat resorption to be caused primarily through simple starvation. The increase in serum phosphatase, which in fact is seen in WEIL and RUSSEL's

curves from the 3rd day, these authors ascribe to a development of new bile ducts, through which bile again can be conducted to the intestine. The present author never observed the development of a new choledochus, and does not hesitate in regarding the increase as a real one, produced by the operation. Furthermore in two of Weil and Russel's curves, at least, the phosphatase, after the transient drop, rises about the 10th day to a level higher than that before the experiment. This has not been satisfactorily explained by these authors. Unfortunately we miss information regarding the duration of the life of the rats in these experiments.

It should be pointed out that the increase in phosphatase which the author has demonstrated in these rat experiments is considerably lower than the corresponding increase in dogs, rabbits and man. Still it is, as demonstrated in a few of the curves in fig. 2 and 3, often lower than the increase produced through fat ingestion, *i. e.* by olive oil.

The author has combined these experiments with histochemical investigations of the localisation of the phosphatase enzyme. The results obtained, which will be published later, indicate that a very considerable production of phosphatase is taking place in the duodenal mucosa, and that the amount of phosphatase here increases during starvation coinciding with the drop in serum phosphatase. Theoretical considerations will be omitted here and lastly it shall only be hinted at, that the duodenum in the rat seems to perform a very conspicuous function during both normal and the pathophysiologic conditions dealt with here.

This work has been supported by "Universitetsstipendiet for helt unge videnskabsmænd" from Aarhus University. My thanks are due also to Professor Bent Andersen, M. D. for allowance to use his Pulfrich photometer, and to A/S Medicinalco, Copenhagen who supplied me the necessary sodium-glycerophosphate.

Summary.

Numerous investigators describe increases of alkaline serum phosphatase in obstructive jaundice in dogs, rabbits and man. WEIL and RUSSEL (1942), however, maintain that rats react with a drop in serum phosphatase. This work has been the subject of much interest in literature.

Series of rats with experimentally ligated bile ducts are fol-

lowed. The surgical technique is described. Controls and sham-operated animals are followed also. For serum analysis a modified LUNDSTEEN-VERMEHREN (1936) method is used, requiring 50 cmm. serum for double analysis. This technique allows comparison with WEIL and RUSSEL's findings.

On insufficiently starved animals uncertain results were obtained. In rats starved 48 hours previous to the operation, which assures constant and minimal serum phosphatase levels a distinct phosphatase increase is shown. This is visible already the day after the operation but it is maximal on the 3rd day or later and is often followed by a slow decrease. (See fig. 2 and 3.)

WEIL and RUSSEL did not use starved rats and the phosphatase drop observed by them is here explained through the anorhoxia following the operation, thus being in reality an inanition effect, which masks the postoperative increase.

The increase in serum phosphatase following bile duct ligation in rats is lower than the corresponding increase known in rabbits, dogs and man and often does not surpass the elevation, which could be produced by feeding, *e. g.* olive oil.

It is hinted at, that the duodenum plays a very important rôle in the regulation of the serum phosphatase in normal rats and in those with experimental obstructive jaundice.

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From the Institute of Legal Medicine of the University of
Copenhagen.

Studies on the Biochemistry of Human Semen.

I. The natural substrate of prostatic phosphatase.

By

FRANK LUNDQUIST.

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The presence in the human prostate gland and seminal fluid of extraordinary amounts of a phosphatase with pH optimum in the acid region was first demonstrated by KUTSCHER and WOLBERGS in 1935. Their investigation was opened with the finding of acid phosphatase in male urine. Later on these authors published a paper on the chemical and physical properties of the enzyme (KUTSCHER and WÖRNER, 1936). Among the animals so far examined for the presence of acid phosphatase in the prostate only monkeys contain amounts comparable to those found in man — about 3 000 units per gram, one unit being the amount of phosphatase that will liberate 1 mg phenol per hour from monophenylphosphate substrate-citrate buffer of specified composition at pH 4.9 and 37° (GUTMAN and GUTMAN, 1940).

Concerning the physiological rôle of this enzyme nothing is known as yet. The considerable concentration of inorganic phosphate reported in semen (GOLDBLATT, 1935, and HUGGINS and JOHNSON, 1933) led the present writer to investigate the possibility that seminal fluid itself might contain a phosphorus compound, which is very rapidly split by the prostatic phosphatase. This was actually found to be the case, and the compound has now been identified as phosphoryl choline. In a short communication preliminary evidence for the presence of phosphoryl choline in human seminal fluid was advanced (LUNDQUIST, 1946).

Experiments with human semen.

The disintegration of phosphorus compounds in semen is extremely rapid as shown by the following experiment:

Ejaculate was collected in a glass container placed in freezing mixture, determination of inorganic phosphorus was made at intervals.

	Inorg. P m% ¹	Total acid- soluble P m%.
1.5 min. after ejaculation, temp about -10°	21.6	110
11 min. (5 min. at about -10° and 6 min. at $+20^{\circ}$.)	45.3	
20 min. after ejaculation	64.2	
34 min. after ejaculation	64.5	

In another experiment the splitting of added glycerophosphate was investigated.

Ejaculate was collected in a glass container cooled in dry ice. About one gram of the ejaculate was used for determination of inorganic P, total acid-soluble P, and acid phosphatase activity. The remaining 5.7 grams were allowed to stand at 20° for 2 hours after which the inorg. P was determined again. 1 ml of the sample was incubated at 37° with 0.5 ml of a 2 % neutral solution of sodium betaglycerophosphate and the inorg. P determined at intervals as shown in the table.

Table 1.

Time min.	Inorg. P m%	inorg. P increase m%	Total acid- solubl. P m%
initial	21.2	0	127
120	108	87	
(temp. 20°)			

1 ml semen + 0.5 ml 2 % glycerophosphate. (Temp. 37°)

0	70.1	0
5	114.5	44.4
10	118.5	48.4
15	132	61.9
20	132	61.9
30	131	60.9

Acid phosphatase activity of the sample: 3 800 U/ml.

¹ The term m% is used to designate thousands per cent instead of the illogical expression mg%. m% is pronounced milli per cent.

Within 15 min. all the glycerophosphate added was split, even though the pH was very far from optimal. When the inhibition caused by the considerable amount of inorg. P present in this experiment is taken into account, it seems quite understandable that this phosphorus compound has escaped notice earlier due to practically complete splitting in the interval ordinarily elapsed between ejaculation and deproteinization.

About 20 ejaculates from two healthy subjects were collected directly in tared glasses containing about 15 ml 10 % trichloroacetic acid, and the inorganic phosphorus determined in the filtrates. Values varying between 10 and 20 m%, were found leaving about 100 m% of the total P unaccounted for.

The properties of the substance in question were investigated through fractionation of the baryum salts. The trichloroacetic acid (5 %) filtrate was neutralized with baryum hydroxide and placed in the refrigerator. The soluble salts were treated with 2 volumes of alcohol, and the precipitate dissolved in water and reprecipitated with alcohol. The water insoluble salts were dissolved in a little trichloroacetic acid, and the procedure repeated. In this way two fractions were obtained, one insoluble in water and the other soluble in water but precipitable by 2 vol. of ethanol. However, by far the greater amount of phosphorus (60—70 %) remained in the alcoholic solution. Further addition of absolute alcohol produced no more precipitate. The water insoluble Ba-salts contained hardly any organic phosphorus compounds. The very small difference between the total P and inorg. P actually found may perhaps be due to adenosinetriphosphate from the sperm cells, as it nearly vanished on heating for 7 min. to 100° in 1 n sulphuric acid. 58 mg of the water soluble—alcohol insoluble Ba-salts were isolated from 5 ejaculates. Analysis showed 1.3 % of org. phosphorus. The substance contained absolutely no reducing compounds as measured with the highly sensitive method of FOLIN (1928). As mentioned later the trichloroacetic acid filtrates are not clear owing to the content of incompletely precipitable proteoses. This contamination may be the cause of the low P content of the alcohol insoluble Ba-salts. The method of KAPLAN and GREENBERG (1944) for fractionation of phosphorus compounds also left most of the phosphorus in the alcoholic solution normally discarded.

The only substances so far found in animal tissues stated to be soluble both in water and alcohol are sphingosine choline phosphate

discovered in kidney by BOOTH (1935) and choline glycerophosphate found in autolysed pancreas by SCHMIDT, HERSHMAN and THANNHAUSER (1945) and simultaneously by KING and ALOISI (1945).

Attempts to purify the unknown phosphorus compound led to the following procedure. The solution containing the alcohol soluble Ba-salts from one ejaculate is concentrated in vacuum to about 10 ml. The solution is clarified through centrifugation, and 90 ml of ethanol added. On addition of a saturated alcoholic solution of mercuric chloride a precipitate is formed. About 10 ml of HgCl_2 is necessary in order to precipitate nearly all the P. The precipitation is completed on standing in the ice box for at least one day. The precipitate is collected through centrifugation and extracted with about 10 ml of water. Most of the substance dissolves and the rest is removed through centrifugation. The mercury is removed from the clear extract with hydrogen sulphide. The solution thus obtained shows a N : P ratio of about 1.2. One or two repetitions of the procedure bring the N : P ratio down to nearly 1.0. This clearly excludes sphingosine choline phosphate, which requires a N : P ratio of 2. Hydrolysis for one hour with 1 n sulphuric acid did not release any measureable amount of choline. This excludes choline glycerophosphate, which according to several authors is a very unstable compound decomposed to glycerophosphate and choline on mild treatment with acids (see *e. g.* SCHMIDT, HERSHMAN and THANNHAUSER, 1945 and KING and ALOISI, 1945). When hydrolysis was carried out in 5 n sulphuric acid for 2 hours 15 % of the phosphorus was split off and choline could be detected in the solution. Heating to 100° with 5 n sodium hydroxide caused slow decomposition with formation of trimethylamine in good agreement with the known properties of choline. In order to effect a total splitting without violent treatment experiments were made with phosphatase, prostatic secretion being used as a powerful source of this enzyme.¹ Rather concentrated solutions of the unknown substance, purified as described above were incubated at 37° with an ample amount (about 0.2 ml) of prostatic secretion for 24 hours. Analyses showed that practically complete splitting had taken place with liberation of equivalent amounts of choline and inorganic phosphate. The same process could be demonstrated in ejaculate as shown by the

¹ Samples of prostatic secretion obtained from patients through digital massage were kindly put at my disposal by dr. FROM HANSEN.

following experiment, where advantage is taken of the circumstance that prostatic phosphatase is inhibited reversibly by fluoride. Ejaculate was collected in 10 ml 1 n sodium fluoride and made up to a total volume of 25 ml. The temperature was 20°.

Table 2.

Time after ejaculation	Inorg. P m%	Choline m%	Equivalent conc. P m%	Total P m%
20 min	21	44.6	10	127
180 min.	45.8	—	—	
Addition of excess CaCl ₂ , 28 hours ...	90.7	420	93.3	

So far, evidence points to phosphoryl choline, the stability against acid hydrolysis indicating a primary phosphorus compound. The properties of this substance have been studied by PLIMMER and BURCH and later by several other authors (BEZNÁK and CHAIN, 1936, and BAER and McARTHUR, 1944). Plimmer and Burch state that the Ca- and Ba-salts are precipitated by addition of one volume of alcohol, though the precipitation is not claimed to be quantitative. Experiments were made in order to settle this point. To 0.1 m (*i. e.* about 3 %) solutions of synthetic calcium phosphoryl choline chloride various amounts of absolute alcohol were added. The tubes were placed in the ice box overnight and centrifugated. Phosphorus determinations were made on the supernatant solution with the following results:

Table 3.

Volumes of alcohol added.	m% P in solution	Corresp. conc. in the aqueous solution
1	129	258
2	18.1	48.3
3	6.0	24
5	0.82	4.9
10	0.33	3.6

It is evident that the precipitation is far from quantitative. The solubility of the Ba-salts was not determined but judging from the paper of PLIMMER and BURCH, 1937, the properties of the Ba-salt are similar to those of the Ca-salt.

As a final proof that the alcohol soluble phosphorus compound in human semen is phosphoryl choline the Ca-salt was isolated and analysed. The purification procedure was similar to that

described above only calcium hydroxide was used instead of baryum hydroxide in neutralising the trichloroacetic acid. Mercury precipitation was performed as usual. The solution of the Ca-salt was neutralised with $\text{Ca}(\text{OH})_2$, evaporated to a small volume (1—2 ml) and clarified through centrifugation. About 10 volumes of absolute alcohol were added with stirring, and the mixture left in the ice box over night. The white precipitate formed was washed with alcohol and dried in the air at 100° . Two ejaculates taken together yielded 23 mg of this substance. The phosphorus content was found to be 8.16 % instead of the theoretical 9.46 % calculated from $\text{C}_5\text{H}_{13}\text{O}_4\text{NPClCa}$, $4\text{H}_2\text{O}$, the composition of calcium phosphoryl choline chloride as given by PLIMMER and BURCH.

4 ejaculates treated in the same way gave a yield of 66 mg of the raw product. The two batches were combined, dissolved in water and filtered after addition of a little charcoal. The solution was concentrated to about 3 ml on the steam bath and 4 volumes of absolute alcohol added. The precipitate thus formed weighed 47 mg after washing with alcohol and ether and drying at 100° for 24 hours.

A sample of calcium phosphoryl choline chloride synthesised according to the prescriptions of PLIMMER and BURCH and dried at the same temperature was analysed simultaneously with the following results:

	P %	Cl %	Ca %	N %
Calculated for $\text{C}_5\text{H}_{13}\text{O}_4\text{NPClCa}$, $4\text{H}_2\text{O}$	9.46	10.75	12.16	4.25
Found for biochem. preparation	9.41	9.47	14.1	4.71
Found for synthetic preparation	9.87	10.6	12.3	4.51

In order to get an impression of to what extent phosphoryl choline accounts for the phosphorus present in human semen analyses for phosphorus and choline were made on samples of semen from persons examined for sterility.¹ The results are shown in tables 4 and 5.

Table 4 shows choline values varying from 172 m% to 398 m% leaving an excess of phosphorus of generally about 30 m% but with large deviations to both sides. In one case a negative value for the phosphorus excess was found. This may be due to an analytical error or perhaps to precipitation of some of the phosphorus

¹ I want to thank drs K. BROCHNER-MORTENSEN and M. FABER for placing the samples from the laboratory of "Sygchuslægerne Organisation" at my disposal.

Table 4.

Determinations of phosphorus and choline on one day old samples of human semen, morphologically normal or only slightly subnormal.

No.	volume ml	sperm count. millions/ml	acid soluble P m%	choline chloride m%	equivalent conc. of P m%	excess P m%
1	5.3	73	74.8	200	44	31
2	3.6	37	67.2	172	38	29
3	4.5	221	139	398	89	50
4	4.3	83	140	271	60	80
5	5.0	?	94	242	54	40
6	2.8	74	82.5	201	44.7	39
7	2.4	56	87.5	262	58.2	29
8	2.2	209	94.3	333	74	20
9	7.0	150	64.3	313	69.5	-5
10	4.3	60	81.6	289	64.3	17

Table 5.

Determination of phosphorus and choline on seminal plasma from one-day-old samples of morphologically normal or slightly subnormal human semen.

No.	volume ml	sperm count. millions/ml	acid soluble P m%	inorg. P m%	choline chloride m%.	equivalent conc. of P m%	excess P m%	Un-split P
1	4.5	57	85.7	72	324	72	0	13.7
2	2.5	105	—	63.9	130	29	34.9	—
3	4.6	22	122	105	397	88.3	26.7	17
4	1.9	53	76.5	49.9	185	41.2	8.7	26.6
5	3 hours after ejaculation.	138	122	543	121	+1	16	
5	30 hours after ejaculation	—	120	548	122	-2	—	
6	3.3	152	90.2	70.3	259	57.5	+12.8	19.9
7	4.0	67	103.	89.1	289	64.2	+24.9	13.9
8	—	—	61.8	42.4	203	45	- 2.6	19.4

in an insoluble form (perhaps spermine phosphate). Table 5 includes determinations of inorganic phosphorus. Considerable fluctuations are seen in the excess of inorganic P. The fraction of the total P present in organic combination was much more constant. The nature of this phosphorus compound or compounds was not investigated. In order to see whether either phosphorus

or choline was used up to any appreciable extent by the sperm cells sample no. 5 was analysed again after 30 hours' standing at room temperature. No significant alterations were found.

Bovine Semen.

As bulls semen is known not to contain excessive amounts of phosphatase a few experiments were made to study the content of phosphorus and choline in bovine semen¹. Table 6 summarises the results.

Table 6.

Determinations of phosphorus and choline on one-day-old samples of bovine semen and seminal plasma.

Bovine, Semen one-day-old samples.

No.	acidso- luble P m%	inorg. P m%	Choline chloride m%	Equivalent conc. of P m%	Excess P m%
1	26.6		31	6.9	19.7
2	41.6		26	5.8	35.8
3	45.2		30	6.7	38.5
4	32.1		31	6.0	25.2
Bovine Seminal Plasma					
5	53.8	13.4	81	18	— 4.6
6	33.4	3.5	85.7	19	—15.5
7	48.0	3.9	27.6	6.13	— 2.2
8	26.6	1.8	34.7	7.71	— 5.9

The total phosphorus and choline content is much lower than in human semen. Numbers 5—8 seem to indicate that in bovine semen choline is not exclusively formed from phosphoryl choline as there is a deficiency of inorg. P. However, these samples were centrifugated, and it is quite possible that some inorganic P is precipitated as an insoluble compound. The samples of table 7 were deproteinised immediately, but there still seems to be a definite excess of choline over that required by the inorganic phosphate. The following experiment was performed in order to see, whether the choline and inorg. P content increases on in-

¹ E. BLOM, veterinary surgeon, has kindly supplied the samples of bovine semen from the laboratory of special pathology and therapy at the veterinary high school, Copenhagen.

Table 7.

*Bovine Semen. Four consecutive ejaculates from the same Bull.
Samples immediately treated with trichloroacetic acid.*

ejaculate	acidsoluble P m%	inorg. P m%	choline chloride m%	equivalent conc. of P m%	excess P m%
1st....		5.1	47.5	10.5	-5.4
2nd ..		3.5	38.5	8.6	-5.1
3rd...	25.4	4.4	22.0	4.9	-0.5
4th...	17.9	3.9	16.0	3.6	+0.3

cupation. Ejaculate was centrifugated immediately after ejaculation. The plasma obtained was stored in the ice box until deproteinisation could be performed (about 4 hours). The remaining seminal plasma was incubated at 37° for 18 hours and analysed again.

	total acid- soluble P m%	inorg. P m%	choline chloride m%	equival. P conc. m%	excess P m%	unsplit P m%
initial values	35.7	4.4	15-20	4	0	31.3
after 18 hours at 37°	—	18.6	108	24	(-5.4)	17.1

In this case there is no doubt that choline and inorg. P were formed in approximately equivalent amount during incubation. Whether the precursor is phosphoryl choline can of course not be decided on this evidence.

Analytical methods.

The phosphorus determinations were made on trichloroacetic acid filtrates. These filtrates were generally turbid owing to the albumoses present in semen, which are only incompletely precipitated. Even in the few cases where clear filtrates were obtained, the determination of inorg. P could not be performed directly, because turbidity developed on addition of the molybdic acid reagent. GOLDBLATT (1935) and HUGGINS and JOHNSON (1933) remove this turbidity through ultrafiltration. In the experiments here reported inorg. phosphate has been precipitated with magnesium and analysed colorimetrically (JANDA and GÖBELL, 1942). On addition of the ammoniacal reagent to the trichloroacetic acid filtrate the turbidity mentioned vanishes completely. Total acid-soluble P determinations were made directly on the filtrates by the method of FISKE and SUBBAROW (1925). Nitrogen was deter-

mined colorimetrically with Nessler's reagent after Kjeldahl-destruction and distillation. Choline was determined by the method of ROMAN (1930) with slight modifications. The precipitate of choline enneaiodide was not collected on a filter but centrifugated down in a de Waard tube and washed with icecold water. The precipitate was dissolved in potassium iodide solution instead of chloroform. A standard choline solution was always analysed simultaneously. For direct estimation of choline in samples of semen deproteinisation with cadmium hydroxide (FUJITA and IWATAKE, 1931) was found very satisfactory, a perfectly clear filtrate being obtained. Experiments with addition of choline to samples of blood plasma showed complete recovery of choline.

All determinations were made in duplicate.

Discussion.

Phosphoryl choline has been isolated from ox liver by INUKAI and NAKAHARA (1935). The yield was, however, very small, only 0.3 g of the picrate being obtained from 200 kg of liver. The rather drastic procedures employed in the isolation and purification of the substance points to the possibility that it was formed during the procedure *e. g.* from lecithin. The properties of synthetic phosphoryl choline have been studied by several authors. It was found to be a very stable compound hydrolysed by acids with about the same velocity as glycerophosphate (BAER and McARTHUR, 1944). Phosphatases from kidney, bone and plasma split phosphoryl choline readily, but choline esterase does not (BEZNÁK and CHAIN, 1936). A very important attempt to elucidate the possible physiological rôle of this substance has been made by RILEY (1944), using phosphoryl choline containing labelled phosphorus. He concludes that it is hardly probable that phosphoryl choline is a precursor of phospholipids, in fact the evidence advanced points to the possibility that the phospholipid catabolism in the liver is inhibited by phosphoryl choline. Another function has tentatively been ascribed to phosphoryl choline, *i. e.* that of giving rise to an active methyl donor on oxidation to phosphoryl betaine (RILEY). This assumption is, however, quite hypothetical.

By far the greater part of the phosphorus content of semen has been demonstrated to originate from the seminal vesicles (HUGGINS and JOHNSON). We therefore infer that phosphoryl choline is formed in these glands. On ejaculation the secretion from the seminal vesicles is mixed with the prostatic secretion,

and a rapid splitting of the phosphoryl choline results. That choline in semen is formed from some precursor through enzymatic activity has been demonstrated clearly by KAHANE and LÉVY (1936) in a little known paper. The phosphorylation of choline is perhaps to be considered a necessary condition for the secretion by the glandular tissue in sufficiently high concentration. At present nothing whatever is known about the possible rôle of choline or phosphorus in the metabolic phenomena related to reproduction. Choline may perhaps be an activator of the fat metabolism of spermatozoa which has been shown to be considerable (LARDY, HANSEN and PHILLIPS, 1945).

I wish to acknowledge the vivid interest which Prof. KNUD SAND has constantly shown this investigation. I am likewise indebted to Miss R. FAHRNER for valuable assistance.

Summary.

The presence of an alcohol soluble phosphorus compound in freshly ejaculated human semen is demonstrated. A purification procedure is described yielding a substance with a N : P ratio of 1 and stable against acid hydrolysis. 46 mg of a white microcrystalline substance was isolated and proved to be the calcium-salt of phosphoryl choline chloride.

Determination of phosphorus and choline were made on one-day-old samples of human semen, where total splitting of the phosphorus compound by the prostatic phosphatase may be assumed. About 80 % of the total phosphorus could be accounted for as inorganic P and phosphoryl choline-P.

Choline is probably secreted from the seminal vesicles only in combination with phosphorus.

Similar experiments on bovine semen showed much lower values of both P and choline. Evidence is advanced for the presence also in bovine semen of a compound liberating P and choline under the influence of enzymic activity.

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Heat- and Cold-sensitive Mammalian Nerve Fibres. Some Somatic Reflexes to Thermostimulation.

By

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All methods by means of which nerve fibres can be differentiated would seem to be of very great interest. Hence, when it was noted, in work on thermostimulation (BERNHARD and GRANIT, 1946), that the cat showed evidence of struggling when limb nerves were locally warmed but remained quiet when they were cooled, it was decided to devote some attention to this mode of stimulation. With an impulse integrator connected to the amplifier BERNHARD and GRANIT had already shown that both local cooling and moderate local warming initiated a discharge but this instrument could not analyze the nature of the discharge obtained. Their intention was to create an 'artificial sense organ', and they succeeded in demonstrating a local drop of potential relative to normal nerve when the temperature was raised or lowered from the level to which the nerve had been adapted. (For earlier work, see their paper, for a full review, C. v. EULER, 1947). GRANIT and SKOGLUND (1945) found that local cooling augmented the transmission through their artificial synapse, formed by the cut end of a mammalian nerve, but that warming, if it had any effect at all, rather tended to diminish the transmitted amount. This experiment too indicated some differentiation between these two modes of increasing the negativity at the treated region.

An extensive and systematic analysis of vegetative responses was now undertaken in this laboratory by C. VON EULER (1947)

whereas we ourselves decided to begin with an inspection of the impulse pattern elicited by local temperature changes to be combined with a preliminary survey of possible somatic reflexes obtainable by this mode of stimulation. Our results will be briefly reported in this paper.

Technique and Procedure.

Some 50 cats were used, nearly all decerebrated, some in addition made spinal by a section in the lower thoracic region. A few animals received chloralose (5 cc per kg of a 1 % solution). The standard technique for impulse recording with cathode ray, loudspeaker and necessary amplifiers was employed. The thermode was a small lacquered metal container with a 1 cm groove for the nerve. In its latest standardized pattern it was made of silver and several times coated with a special isolating material, each fresh coating dried at 300° C for several hours. This secured the perfect isolation necessary for the recording of thermopotentials. Our analysis of thermopotentials falls outside the scope of this paper.

The thermode contained a thermocouple, recording the temperature of the water circulating through it, and connected to a moving coil Multiflex mirror galvanometer, critically damped to a period of 1 sec. Its deflexion was projected vertically on the film. In order to preserve a reasonable sensitivity without increase of film width the galvanometer mirror was illuminated by two adjacent light points in such a manner that when a large deflexion removed the one light spot from the film the other light spot took over and continued the temperature record (see figs. 1, 3 and 4). The sensitivity was then 1.3—1.6 mm per degree C, depending upon the thermocouple used.

For reflex work the standardized denervated decerebrate preparation of the SHERRINGTON type (see CREED *et al.*, 1932) was used. For myographic recording *m. tib. ant.* or *m. gastroc.* (not isolated from *m. soleus*) was freed and connected to the isometric lever of the spring of a Brown-Schuster myograph, fitted with two crossing coils one of which was fed by a high frequency circuit. Since the one coil remained fixed and the other altered its position relative to the former with the deflexion of the myograph spring, capacitative changes were set up by the isometric muscle contraction, and these were transmitted to a cathode ray oscillograph by means of an amplifier. In this manner a vertical record of the reflex response was filmed together with the thermode temperature. The impulses in the nerve or in the muscle were recorded horizontally by a sweep circuit deflecting another cathode ray, generally every 1.2 sec. During the relatively slow warming and cooling of a nerve, samples of its impulse activity could thus be inspected at every desirable sweep speed without an inconvenient vertical expansion of the temperature and muscle records owing to high film speeds.

The cat was kept in an isolated box in steam at about 25—30° C.

Results.

1. *Impulse records from root filaments in response to thermostimulation of the sciatic.*

Some 50 filaments, nearly all from the sensory roots L7 or S1 (since motor effects could be observed myographically), were isolated as thin as possible, listened in to, and the discharge photographed. For consistent results the following precautions have to be observed: (i) the thermode must not be placed too near a cut or crushed region of the nerve since this is hypersensitive, as is well known from work by ADRIAN (1930) and SKOGLUND (1942). (ii) It is necessary to make certain, by root sectioning and denervation, that the impulses recorded do not come from proprioceptors activated by reflex or direct muscle contractions elicited by thermostimulation. (iii) The cat must not be hyperventilated. Hyperventilation is well known to increase excitability. Recently KUGELBERG (1944) has measured the drop in rheobase and accommodation and studied the sensory paresthesias set up by hyperventilation.

If these precautions be observed, it is found that the typical response to warming (keeping below 45° so as not to damage the nerve) is difficult to obtain whereas in most preparations large spikes come bursting when the nerve is cooled. The response to heat is a slight increase of noise level, better heard than seen.

Three records to warming are shown in fig. 1 a, b and c. The level at which effects appear depends somewhat upon the level of previous thermal equilibration. The records are seen to consist of small fibre activity, undoubtedly a considerable proportion of unmyelinated C-fibres in addition to small myelinated ones. The responses to cold, fig. 1 d and e, the latter at lower amplification, are very different. In the former there is also a characteristic off-response when the temperature swings back to normal, unusually brisk in this particular case. Such off-effects should not be mistaken for heat responses which start at temperatures above 37° . This mistake is easily avoided if warming is preceded by a period of equilibration removing off-effects. Quite often the latter are missing.

Fig. 1 e shows that one often obtains filaments with a large number of clearly distinguishable spikes of different size. We expected that in such cases it sometimes would be possible to

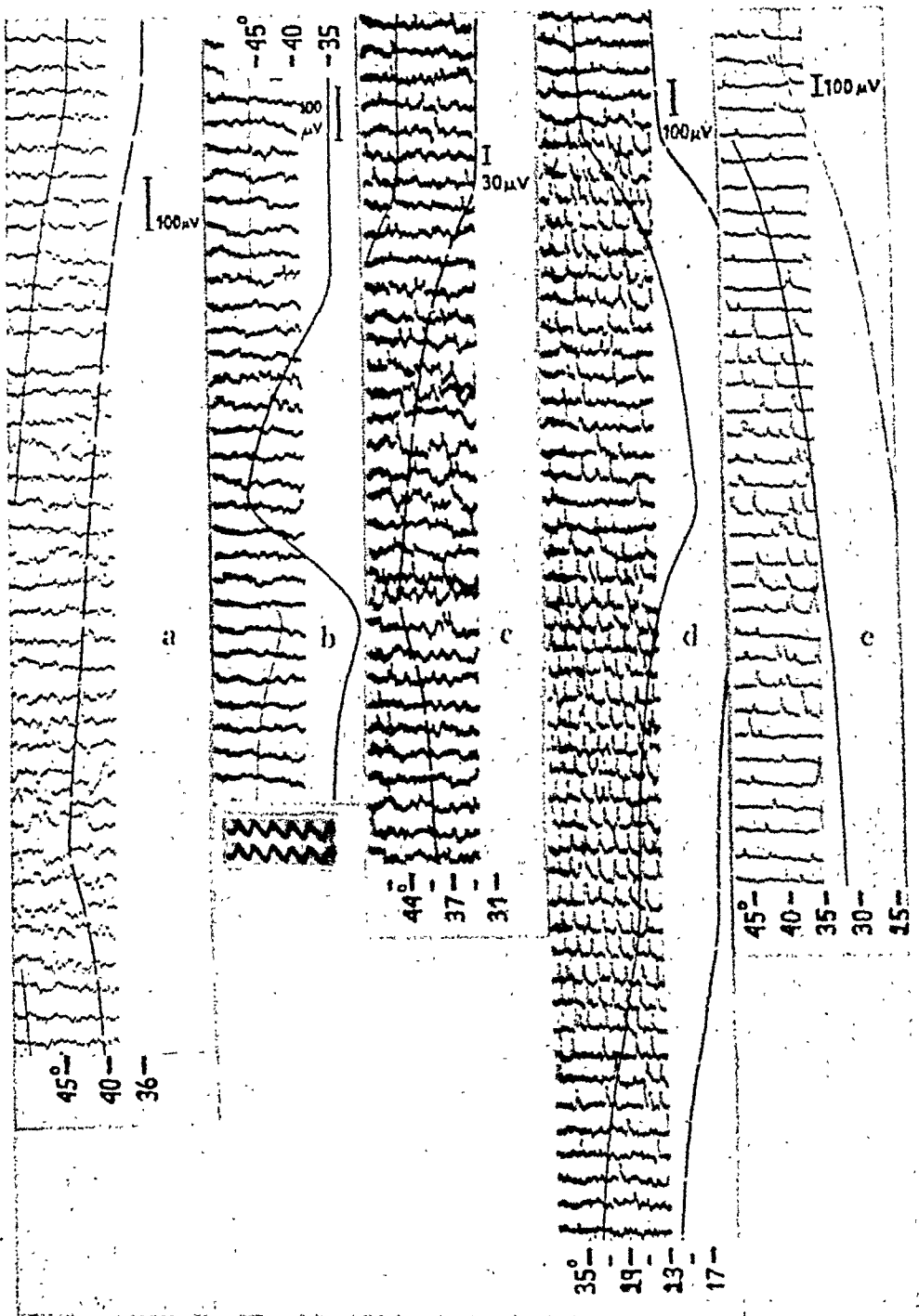


Fig. 1. To be read downwards. Records from thin filament strands of sensory roots. Stimulation by thermode on the sciatic nerve. *a*, *b* and *c* in response to warming. Thermode temperature recorded by parallel black lines one of which interrupted every second. Temperature scale refers to that particular line which embraces the significant range. *d* and *e*, same in response to cooling. Calibration of cathode ray marked on each film, sweep speed by separate record of period 100 Hz.

find spikes which were activated both by cooling and warming. But we have not succeeded in demonstrating the expected overlapping of heat- and cold-sensitivity though other experiments suggest that it might exist. There must, at any rate, be a relatively insensitive zone between the smallest heat-sensitive and the largest cold-sensitive fibres.

In good agreement with these results is the absence of a motor effect to heat. Occasionally a just perceptible response is seen, but the effect is very rare. Heat might nevertheless stimulate the small fibres of the motor roots. LEKSELL's (1945) careful analysis in this laboratory of the motor gamma and delta fibres has shown very definitely that these fibres do not cause myographically measurable contractions. Their effect can only be observed indirectly as an increase of activity in the proprioceptive fibres, picked up in the sensory roots. For the time being the possibility that heat might stimulate these fibres must be left open.

Cold, however, does stimulate the motor fibres but the effects are generally much smaller than a maximal tetanus, unless there is evidence of hyperexcitability due to forced breathing. On the whole it seems as if the afferent fibres were a great deal more sensitive to thermostimulation than the efferent ones. Accommodation, which is greater in the motor than in the sensory fibres of the cat (SKOGLUND, 1942), may play a rôle in determining the magnitude of the effect.

C. v. EULER's extensive work (1947) with the same type of apparatus and mode of stimulation has demonstrated that warming elicits a very strong blood pressure reaction combined with the other components of the general reflex pain response: panting, struggling, pupil dilatation etc. Cold does not elicit this characteristic response pattern. Occasionally he noted a blood pressure increase to cooling but these cases were very rare. Within the efferent vegetative system responses to thermostimulation are practically non-existent. Some idea about the overlap may be obtained from C. v. EULER's experiments on the rabbit's depressor (fibres below about 5μ) in which a strong depressor reaction is elicited by warming and a small one by cooling. Size, on the other hand, need not be the sole determinant of the effects found in different types of nerve in different animals, though it gives a good first approximation. More experience is needed for detailed differentiation.

Nevertheless, it is desirable to summarize our present state of knowledge of this interesting property of nerve fibres in a tentative diagram such as that of fig. 2, to be modified and improved as experiences multiply. The region of overlap is placed around 5 μ . The electrical records and the very strong blood pressure responses suggest a large component of C-fibre activity (RANSON and BILLINGSLEY, 1916; ZOTTERMAN, 1933; CLARK, HUGHES and

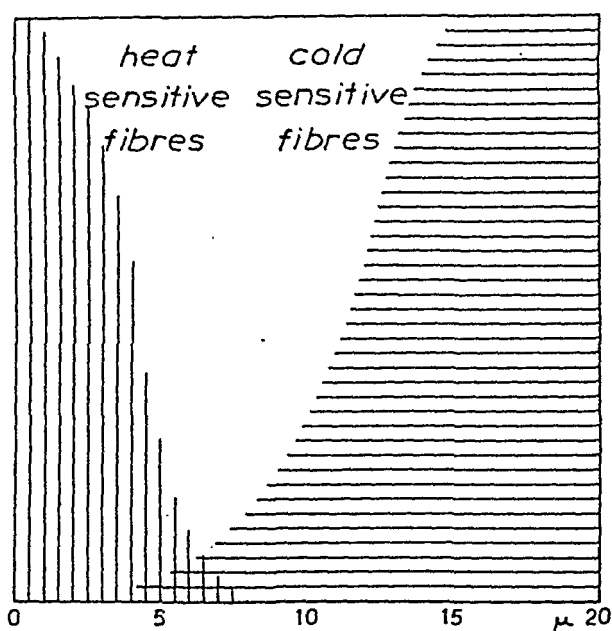


Fig. 2. Diagram illustrating heat- and cold-sensitive nerve fibres.
Abseissae: fibre diameter in μ .

GASSER, 1935) but it will be remembered that blood pressure responses, respiratory reactions etc. also are obtained by stimulation of δ -fibres (HEINBECKER, BISHOP and O'LEARY, 1933).

We have reasoned as if the difference between the excitatory effects to cold and heat were altogether due to differences of excitation without mentioning the possibility that cold may inhibit impulse generation in small fibres and heat, *vice versa*, in large fibres. For the former view there is no evidence but from time to time we have seen a spontaneous discharge in large fibres be inhibited by warming. Occasionally one also finds a half maximum alpha spike sent through a warmed region to diminish in size. A record of inhibition of a spontaneous discharge is shown in fig. 3. Such records suggest that the mechanism of

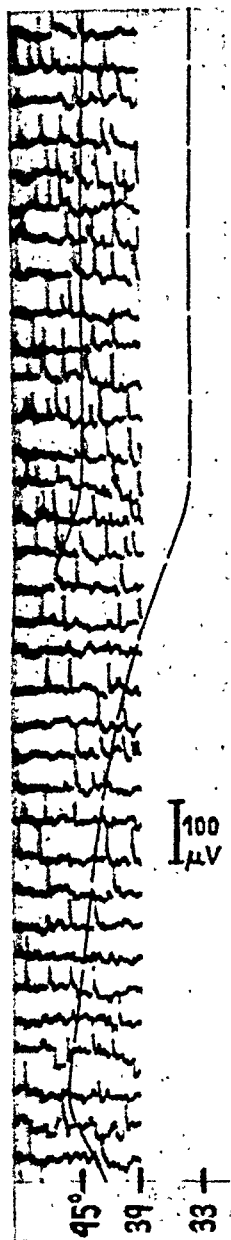


Fig. 3. Spontaneous discharge, picked up in thin strand of root filament, is diminished by warming thermode on sciatic. To be read downwards. Marked as fig. 1.

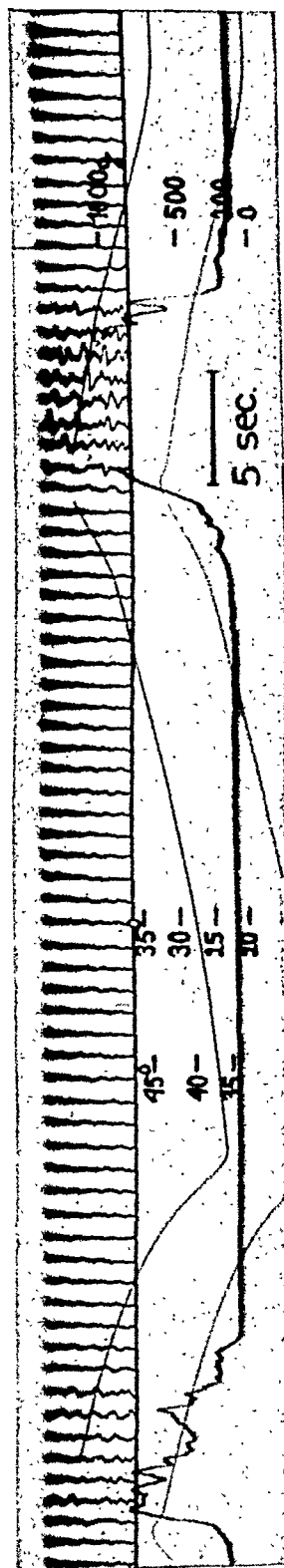


Fig. 4. The tibial muscle responding reflexly to thermostimulation of the ipsilateral popliteal nerve. Silver pins in the muscle record spike activity on vertical sweep (upper half). Temperature as in fig. 1. Note, separate scales for the two lines. Remaining record, myograph.

selectivation might be quite complex. BERNHARD and GRANIT (1946) also noted that warming sometimes inhibited a spontaneous discharge from the cut end. They did not at that time realize that heat and cold excite different fibres. BREMER and TITECA (1946) report that in frog nerves, uniformly heated in a paraffin bath, the large fibre component of a response to a shock disappears.

2. *Some somatic reflexes in response to thermostimulation.*

The flexor reflex. In fig. 4 muscle contraction and thermode temperature are recorded vertically, the electrical activity in the *tib. ant.* muscle (with large silver pins) horizontally. The thermode was applied to various nerves, generally to the popliteal branch of the sciatic. It is seen that warming elicits a strong reflex contraction throwing the myograph out of focus and out of its proportionality range whereas cooling has no effect whatsoever. The record is typical. Cold responses are, however, sometimes obtained. We conclude from our observations on the spinal cat that an ipsilateral flexor reflex is easily elicited by heat but difficult to excite, or at least less regularly excited by cold.

If some other nerve twig be used for simultaneous stimulation with electrical shocks, cooling may facilitate the synchronized reflex responses set up in this manner. The present technique, planned for a general survey only, is not suitable for following up such observations. The result is mentioned in order to draw attention to the significance of reflex background. Assuming cold to stimulate a few fibres of medium size, capable of setting up a flexor reflex, it will depend upon the background whether the effect becomes visible or not.

SHERRINGTON (1910) distinguished two types of flexor reflexes, one that was a component in locomotion, another of nociceptive character requiring stronger stimulation, probably therefore smaller fibres. Our impulse records combined with v. EULER's results suggest that local warming of a nerve is a fairly selective stimulus for the nociceptive flexor reflex, a fact that would seem to be of methodical interest from various points of view.

A crossed extensor. This reflex was studied in decerebrate cats, de-afferented on the side of the muscle tested so as to remove the myotatic appendage. The results were very uniform. Cold did not elicit any effects, although it sometimes facilitated a simultaneous electrically initiated crossed extensor reflex. But heat applied to the opposite sciatic nearly always excited a good

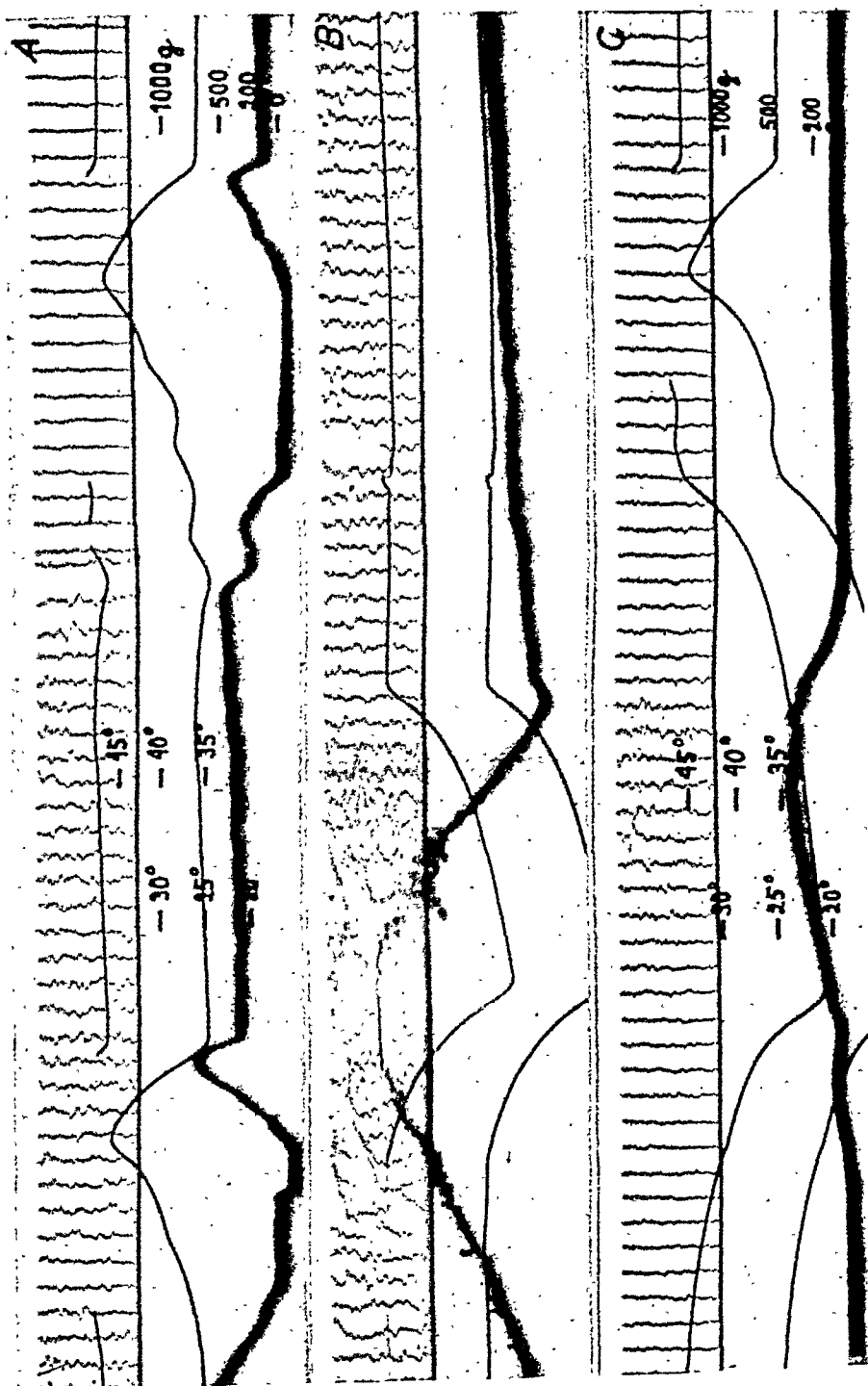


Fig. 5. Reflex and direct response of the gastrocnemius muscle to thermal stimulation of its own nerve. Marked as fig. 4. A. Warming of nerve, twice in succession. B. Directly continued from A but this time response to cooling. C. Response to cooling of nerve to opposite gastroc. after deafferentation.

crossed reflex in the gastrocnemius muscle, thereby placing it in the small fibre range. Our results do not, of course, exclude the possibility that electrical synchronized volleys in large fibres could elicit crossed extensor reflexes but stimuli of this type are always more artificial than the slowly rising discharges set up by thermostimulation.

Reflexes with afferent and efferent path in the same muscle nerve.

This case is particularly well suited for a method that picks out the afferent small fibres without concomitant effects on the motor supply. Responses to heat will thus practically always be pure reflex in character whereas responses to cold will be combined, reflex *plus* direct motor. Theoretically it is simple enough in such cases to cut the motor nerve high up in the thigh after the test with cold, repeat the experiment and subtract the direct motor component. In actual practice this method is not always reliable since the response to thermostimulation rapidly deteriorates, particularly that to cooling, and so errors arise in estimating the reflex fraction of a contraction to cold. A more satisfactory way is to use the other side de-afferented and try the symmetrical muscle on that side as control. Both procedures have been employed.

In fig. 5 we can inspect the course of an experiment in which the thermode was on the medial and lateral *nerves to the gastroc. muscle*. It begins with record A, directly continued in B. It is first seen that heat elicits a small contraction followed by complete inhibition to the myograph zero. A rebound (hardly started as an off-effect in the nerve, since off-effects have only been seen after cooling as in fig. 1) follows the return of the temperature to the level of equilibration after which heating is repeated with the same outcome as before. Continuing the experiment in B we find cooling to begin at the moment when the rebound contraction has diminished so that the muscle again has approached its initial level of moderate tension, though the electromyogram indicates greater activity. The effect of cooling, both in the myograph and the electrical record, is so strong as to upset the whole system of recording. Record C shows the control of the pure motor component obtained with the de-afferented corresponding pair of gastroc. nerves on the other side of the animal. Heating causes no effect whatsoever, cooling a contraction of some 400 grams only.

The very characteristic inhibition to warming has only been seen in decerebrate cats, not in spinal ones. Hence it can hardly have been a peripheral inhibition in the nerve itself. Otherwise the result is typical in illustrating a large-fibre reflex to cold, probably coming from the large proprioceptors of the muscle spindles (SHERRINGTON, 1894) and a mixed response to heat initiated by small muscle afferents. Among known inhibitory effects in extensor reflexes upon the acting muscles themselves should be mentioned SHERRINGTON's well-known 'lengthening reaction' as well as the general effect noted by LEWIS (1942) upon injection of NaCl-solution in a muscle (see also, GELLHORN and THOMPSON, 1945).

M. tib. ant. responds reflexly to warming of its own nerve by contracting. The inhibitory component, which we found above in gastroc.-gastroc. stimulation with heat, does not appear, so that excitation must dominate for the flexors. The response to cooling is less regular, nevertheless often seen, and partly reflex in character. There are, of course, large afferent fibres in the flexors too (REXED, 1947).

In general the reflex thermoeffects are less regular than reflexes set up electrically and hence by synchronized impulses. The asynchronous thermal discharge has to withstand the full influence of slow accommodative counterprocesses and, besides, the thermally initiated impulses do not support each other by electrical forces in the same manner as those set up simultaneously.

Discussion.

As a complement to and in combination with the purely electrical methods of analysis thermostimulation may well prove to be of value in reflex work. A disadvantage in the methods utilizing electrical shocks is that one always is forced to pass from large to small fibres, when increasing stimulus intensity, and that it therefore is impossible to stimulate small fibres selectively without recourse to complicated and tedious methods of blocking of the larger ones. A reflex background of small fibre activity can easily be introduced by moderate local warming of a suitable nerve. We hope to return to this aspect of our work later.

Very intriguing is the question as to what constitutes this rather striking difference in sensitivity to heat and cold. BERN-

HARD and GRANIT (1946) thought that both heat and cold stimulate by means of the generator potential, set up by the thermic stimulation. In fact, they were led to their work by this particular and so far profitable theory. If this view is right it means that the heat and cold potentials, though of the same electrical sign, are set up in different fibres. C. v. EULER (1947) has made the valuable discovery that among the nerves studied by him some responded with a local drop of potential to heat, others to cold, others again to heat and cold. This will no doubt prove significant. We shall return to this aspect of the problem in another paper. The irregular inhibition of large fibres by heat suggests that selective stimulation of the small fibres by warming may be supported by a simultaneous depression in the large fibres. ZOTTERMAN (1936) has reported that *adequate* stimulation of the small fibres mediating temperature sensations may inhibit simultaneous excitation of the touch fibres, as when, for instance, a drop of cold or hot water is dropped onto the tongue (cat). The touch response may then be suppressed so that the temperature response appears alone.

Summary.

Records from thin strands of root filaments show a selective small fibre response to local warming and a selective large fibre response to local cooling of a nerve (cat) with a thermode.

The response to cooling is often accelerated into an off-effect when the thermode temperature suddenly swings back to normal.

The heat response is only seen in afferent fibres, the response to cooling is found in both efferent and afferent nerves, but better developed in the latter.

This being the case heat can be used for stimulation of the small-fibre component of a muscle nerve in order to study the reflex effect on its own muscle. This method has been applied for an analysis of such reflex responses upon *m. tib. ant.* and *m. gastroc.*

It has further been found that a flexor and a crossed extensor reflex are easily elicitable by heat but less regularly by cold.

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On the Utilization of Peptide Bond Amino Acids by Lactic Acid Producing Microorganisms.

By

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During the last year the author adapted microbiological methods for the determination of the amino acid composition of the hormone secretin. When comparing the hydrolyzation methods of proteins used by the workers in this field it was found that the conditions differed considerably (cf. SMITH et al. 1946, GREENHUT et al. 1946, DUNN et al. 1944, HIER et al. 1946, STOKES et al. 1945). The evidence of a complete hydrolysis usually consisted in the agreement of microbiological amino acid values obtained after a certain time of hydrolysis. In a recent paper the hydrolyzation of proteins by mineral acids was exhaustively investigated by LUGG (1946), and when comparing the conditions for a complete hydrolysis given by him with some of the methods used by the workers mentioned above there seemed to exist possible discrepancies (cf. also HAC et al. 1945, SCHEIN and BERG 1943). Some of the hydrolyzation methods used in the microbiological work were controlled by Van Slyke analysis and it was found that a complete splitting of the peptide bonds was not always obtained. This result raised the question if the microorganisms used for the microbiological amino acid determinations could utilize peptide bond amino acids as well as the corresponding free acids. This problem was investigated by means of a series of synthesized valine and leucine peptides.

Experimental.

The assay technique given by STOKES et al. (1945) for the determinations with *Streptococcus faecalis* and *Lactobacillus delbrückii* LD5 was followed. *Lactobacillus casei* was grown in the medium described by McMAHAN and SNELL (1944). Separate solutions of basal media, standard amino acids, peptides and inocula were delivered to 6 inch test tubes. The total volume in each case was 6 ml. The standard and peptides were each run at four levels and from five to six tubes were employed at each level. By using numbers of tubes assay values of relatively high accuracy were obtained (cf. DUNN et al. 1945).

The peptides were synthesized according to the FISCHER (1906) procedures and were checked with respect to total nitrogen, amino nitrogen, ash and moisture. The following peptides were used: glycyl-leucyl-glycine (GLG), glycyl-leucyl-alanine (GLA), alanyl-leucyl-glycine (ALG), leucyl-glycine (LG), glycyl-leucine (GL), alanyl-leucine (AL), leucyl-alanine (LA), glycyl-valine (GV), valyl-glycine (VG), and valyl-alanine (VA). In the microbiological standardizations the leucine and valine peptides were used in amounts calculated with regard to their content of l-leucine and l-valine using the same levels as in the standard curves. The assays were titrated with 0.05 N NaOH.

When the leucine peptides were assayed with *Streptococcus faecalis* it was found that leucine in the tri-peptide glycyl-leucyl-alanine was not available for the microorganism and the leucine

Table 1.

Results of Assays of Leucine Peptides with *Streptococcus faecalis*.

The values are the titration volumes of 0.05 N NaOH.

Substance	Amount of amino acid per tube				
	60 γ ml	30 γ ml	15 γ ml	6 γ ml	0 γ ml
Leucine					
Glycyl-leucyl-glycine	8.21	7.85	5.80	3.39	0.40
Glycyl-leucyl-alanine	0.59	0.50	0.51	0.45	—
Alanyl-leucyl-glycine	8.25	6.50	4.90	3.10	—
Glycyl-leucine	9.80	7.85	6.06	3.45	—
Leucyl-glycine	9.45	7.95	6.10	3.50	—
Alanyl-leucine	9.50	7.90	5.90	3.50	—
Leucyl-alanine	4.08	2.80	1.10	0.60	—

Complete medium 10.07.

equivalent of the di-peptide leucyl-alanine only partially. A comparison seemed to demonstrate that only the combination of leucine and alanine in this order was not utilized. (Table 1.)

Next the valine peptides were tested with *Streptococcus faecalis* and *Lactobacillus delbrückii* LD5 and also in these series the combination of valine and alanine in this order was incompletely utilized. (Table 2.)

Table 2.

Results of Assays of Valine Peptides with Streptococcus faecalis and Lactobacillus delbrückii LD 5.

The values are the titration volumes of 0.05 N NaOH.

Substance	<i>Streptococcus faecalis</i>					<i>Lactobacillus delbrückii</i> LD5					
	Amount of amino acid per tube										
	60 γ ml	30 γ ml	15 γ ml	6 γ ml	0 γ ml	60 γ ml	30 γ ml	15 γ ml	6 γ ml	0 γ ml	
Valine	9.80	8.04	6.21	3.80	0.61	8.10	6.75	4.0	2.81	0.18	
Glycyl-valine ..	9.85	7.25	5.25	3.45	—	6.75	4.50	3.65	2.50	—	
Valyl-glycine ..	10.05	9.0	6.80	3.95	—	8.06	5.10	3.75	2.65	—	
Valyl-alanine ..	3.17	2.70	2.10	1.50	—	4.50	2.03	1.40	0.55	—	
Complete medium					11.80	Complete medium					8.80

The series of leucine peptides assayed with *Lactobacillus delbrückii* LD5 showed that this microorganism did not use leucine in the tri-peptide glycyl-leucyl-glycine at all, and the leucine part

Table 3.

Results of Assays of Leucine Peptides with Lactobacillus delbrückii LD5.

The values are the titration volumes of 0.05 N NaOH.

Substance	Amount of amino acid per tube				
	60 γ ml	30 γ ml	15 γ ml	6 γ ml	0 γ ml
Leucine	9.53	6.31	3.42	1.98	0.95
Glycyl-leucyl-glycine	1.05	1.00	0.95	1.00	—
Glycyl-leucine-alanine	9.00	6.04	3.34	1.80	—
Alanyl-leucyl-glycine	9.23	6.90	4.80	2.10	—
Alanyl-leucine	9.30	7.15	4.58	1.95	—
Leucyl-alanine	9.10	6.34	3.25	2.0	—
Leucyl-glycine	6.19	3.10	1.75	1.05	—
Glycyl-leucine	8.50	5.80	3.20	1.90	—

Complete medium 12.50.

of the di-peptide leucyl-glycine only partially. The comparison of the results of this series demonstrated that the combination of leucine and glycine in this order seemed to be rather resistant to the action of the microorganism. (Table 3.)

In the series of experiments with *Lactobacillus casei* it was found that this microorganism completely utilized all of the tested leucine and valine peptides. (Table 4.)

Table 4.

Results of Assays of Leucine Peptides with Lactobacillus casei.

The values are the titration volumes of 0.05 N NaOH.

Substance	Amount of amino acid per tube				
	60 γ ml	30 γ ml	15 γ ml	6 γ ml	0 γ ml
Glycyl-leucyl-glycine	11.0	8.10	5.43	2.25	1.20
Glycyl-leucyl-alanine	10.58	6.50	4.10	1.90	—
Alanyl-leucyl-glycine	11.01	8.25	5.52	2.10	—
Leucine	10.48	8.0	5.35	2.22	—
Alanyl-leucine	11.20	8.75	5.65	2.25	—
Leucyl-glycine	10.01	7.85	5.24	2.12	—
Leucyl-alanine	11.32	8.90	6.05	2.30	—
Glycyl-leucine	10.62	8.74	5.65	1.95	—
Valine	9.74	7.98	6.50	4.10	—
Valyl-glycine	9.93	8.25	6.20	4.00	—
Valyl-alanine	9.10	6.50	4.50	3.80	—
Glycyl-valine	9.70	6.72	4.85	3.50	—

Complete medium (leucine assays) 11.81

Complete medium (valine assays) 11.98

Discussion.

The present investigation confirmed the assumption that lactic acid producing microorganisms only are capable of using some of the essential amino acids supplied in peptide form to the basal medium when they are incorporated in certain orders in the peptides. It therefore seems advantageously to follow the progress of protein hydrolysis by chemical analysis since a control of the hydrolysis only by a serial microbiological amino acid assay may be impaired by certain errors. The amino acids, valine and leucine, specially investigated in the present work were chosen since it has recently been shown that enzyme resistant peptides may contain these amino acids (ÅGREN 1947). The use of a partially hydrolyzed protein material in the microbiological assay

Table 5.

Qualitative Results of Assays of Leucine and Valine Peptides with Streptococcus faecalis, Lactobacillus delbrückii LD5 and Lactobacillus casei.

++, +, and 0 signify maximum growth, half maximum growth respective no growth effect of the peptides when compared with the effect of the corresponding amounts of free l-form of the two essential amino acids. Abbreviations are explained in the experimental section.

Microorganism	P e p t i d e s									
	GLG	ALG	GLA	GL	LG	LA	AL	VA	GV	VG
<i>Streptococcus faecalis</i>	++	++	0	++	++	+	++	+	++	++
<i>Lactobacillus delbrückii</i> ..	0	++	++	++	+	++	++	+	++	++
<i>Lactobacillus casei</i>	++	++	++	++	++	++	++	++	++	++

methods may possibly be complicated by other errors. ANGIER et al. (1946) and WOLLEY (1946) have demonstrated that naturally occurring peptides or peptides formed during enzyme hydrolysis also may possess special microbiological growth properties not directly associated with their content of amino acids. During the experimental conditions used in the present investigation our peptides did not show any such effects.

The result of the analysis with leucine and valine peptides may also be evaluated in another way. In table 5 the growth data are qualitatively collected.

It seems obvious that this type of analysis in combination with chemical methods may be further developed to identify the order in which the amino acids of peptides are linked together. The inherent property of most of the lactic acid producing microorganisms to use only the natural antipode of an amino acid may also simplify some types of such analysis.

Summary.

Streptococcus faecalis, *Lactobacillus delbrückii* LD5 and *Lactobacillus casei* are capable of using the essential amino acids leucine and valine supplied in peptide form to the basal medium only when they are incorporated in certain orders in the peptides. These results stress the weight of a chemical control of the progress of the protein hydrolysis when working with microbiological amino acid assay technique. The results of the investigation may

be further developed to identify the order in which the amino acids of peptides are linked together.

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Microestimation of Electrolytic Conductivity.

By

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In biological work it is often important to be able to obtain a rapid evaluation of the concentration of the sum of inorganic electrolytes in small samples of fluids containing salts.

Estimation of the electrical conductivity is undoubtedly the quickest procedure available for this purpose, although because of technical difficulties it has not hitherto been very much used of a micro analysis.

The aim of the present study is to introduce a very simple procedure suitable for determination of the electrical conductivity of small samples of biological fluids, and salt solutions.

The sample to be analysed is delivered into a measured quantity of distilled water previously introduced into a small conductivity vessel. The resistance to alternating current is now measured in proportion to the same degree of dilution of a known NaCl-solution (*e. g.* a solution containing 150 mM. NaCl/litre) placed in a similar electrode vessel. Fig. 1.

Making use of this arrangement it should within certain limits be permissible to neglect temperature, electrolytic polarisation, conductivity of the distilled water used for dilution, the retarding effect of proteins and the activity coefficients of the fluids. Furthermore precise calibration of each of the electrode vessels and of the pipettes used for dilution should be superfluous.

The electrode vessels (Fig. 2) are made so equal to each other as possible. The electrodes, each about 0.8 cm² consisting of platinum foil, are sealed on to the inner surface of a small tapered glass vessel, and the dimensions adapted so that the resistance between the electrodes reaches a minimum when the volume of

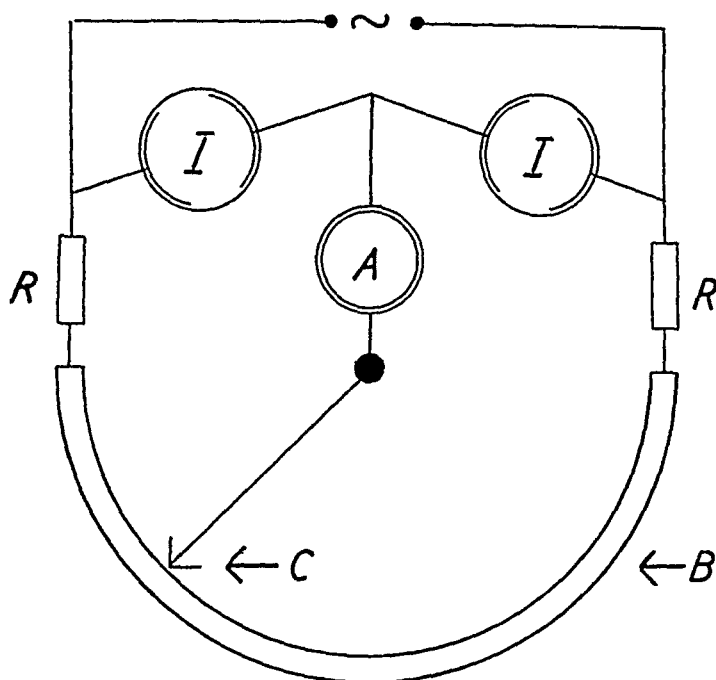


Fig. 1. Wheatstone-bridge arrangement.

I and II: electrode vessels.
 A: indicator of equilibrium of the bridge.
 R: built-in resistances.
 B: resistance wire and dial.
 C: sliding contact.

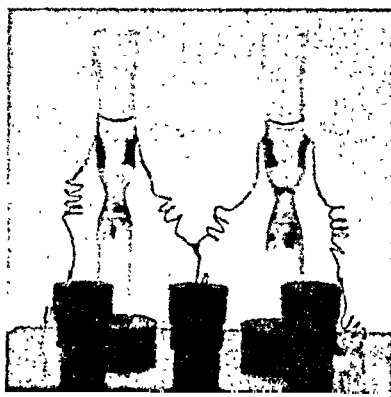


Fig. 2. Photograph of the electrode vessels.

fluid contained exceeds about 0.9 ml. In order to make it possible to perform the measurements at 50 Hz the electrodes should be coated with a thin layer of platinum black. This was done by

placing a solution of platinum chloride, H_2PtCl_6 , $6\text{H}_2\text{O}$ (about 2.5 per cent), containing 0.02 per cent lead acetate in the vessels. The electrodes of the vessels were connected in series and a voltage of 4.5 volts were applied for 20 minutes, the current being reversed every 2 minutes.

The vessels can easily be emptied by means of a capillary glass tube connected to a water suction pump. The dimensions of this glass tube are adjusted to let a volume of about 1 ml be removed in not less than 4 seconds. — After being used, the vessels are washed twice with 1 ml distilled water. When, in the course of months, they become greasy they are cleaned by means of a solution of 5 per cent sodium dichromate in concentrated sulphuric acid left in the vessels overnight. When not in use the vessels should be filled with distilled water and covered with a glass lid.

For the resistance measurements a fairly simple commercial Wheatstone-bridge arrangement, "Philips Philoscope" (G. M. 4140), is used. In this instrument the equilibrium of the bridge is indicated by a small electronic ray tube.

The instrument is provided with built in standard resistances allowing measurements of unknown resistances between 10^{-1} and 10^{+7} ohms with an error, which for the individual bridge used by the author, did never exceed 2 per cent, which is about twice the maximum reading error. The instrument may as well be used as an "open bridge" allowing comparative measurements. The difference between resistances not differing more than \pm_{20}^{25} per cent can be read directly from the dial expressed in per cent of one of the resistances compared. In this measurement the error did not exceed 0.2 per cent of the smallest of the resistances.

The "Philoscope" may be fed with alternating current of 50 Hz and about 2 volts through a built in transformer. However, the measurements underlying the working out of the present method were carried out at 1 000 Hz also. In these experiments the currents of 1 000 and 50 Hz were obtained from the "Philips" oscillator G. M. 4260.

Before using the arrangement for determinations of the conductivity of unknown solutions the relative capacity of resistance of the electrode vessels should be determined.¹ — The vessels

¹ The capacity of resistance of a conductivity vessel is the product of the known specific conductivity of some standard solution and the measured resistance of the same fluid. The specific conductivity of an unknown fluid is found by dividing the resistance measured into the capacity of resistance of the vessel used.

previously filled with distilled water are emptied and exactly equal volumes of distilled water, about 1 ml, run into each of them. The syringe pipette of Krogh (1935) is specially suitable for this purpose owing to its extremely precise delivery. But of course the individual syringe used for distilled water should never be used for any other fluid. Now equal quantities of some salt solution, *e. g.* 0.1 ml of a 150 mM. NaCl-solution is delivered into each vessel. For this delivery may be used an 0.1 ml Krogh syringe pipette or a semiautomatic Carlsberg pipette as described by LEVY (1936). The fluids should be mixed either by means of a stream of air (not expired air) through a fine capillary tube or by rotating a small glass spatula in the fluid for a few seconds. The sliding contact of the "Philoscope" is now turned to the position where the electronic-tube indicates balance of the bridge, and a figure, p_0 , indicating the percentage to be added to the resistance of the vessel I to give the resistance of vessel II is read from the dial. Repeating the procedures described, the differences between the readings should not exceed ± 0.2 per cent.

We call the capacity of resistance of the two vessels k_1 and k_2 respectively, and we have the specific conductivity

$$\kappa = \frac{k_1}{r_1} = \frac{k_2}{r_1 + \frac{p_0}{100} \cdot r_1} \quad \text{I}$$

so

$$k_1 = k_2 \cdot \frac{1}{1 + \frac{p_0}{100}} \quad \text{II}$$

We are now able to determine the ratio between the conductivities of two different solutions.

The procedure is the same as described for the adjustment of the cells, but now the known NaCl-solution is placed in vessel II only. In vessel I is placed a sample of the fluid to be measured, using the same syringe or pipette as applied for the standard NaCl-solution. If a pipette is used for this delivery it should be washed out twice with the solution in the vessels in order to give precisely the same delivery of different fluids. — After mixing, the sliding contact is turned until equilibrium of the bridge, and a figure p_x read which indicates the percentage to be added to the resistance of vessel I, to give the resistance of vessel II.

We are now able to figure out the ratio between the conductivities κ_x and κ_k of the fluids in the two vessels. We have

$$\kappa_x = \frac{k_1}{r_1} \quad \text{III}$$

applying II we may write

$$\alpha_x = \frac{k_2}{r_1 \left(1 + \frac{1}{100} p_0\right)} \dots \dots \dots \text{IV}$$

and

$$\alpha_k = \frac{k_2}{r_1 \left(1 + \frac{1}{100} p_x\right)} \dots \dots \dots \text{V}$$

from IV and V we get

$$\alpha_x = \alpha_k \cdot \frac{100 + p_x}{100 + p_0} \dots \dots \dots \text{VI}$$

As p_0 for the couple of vessels used in this study, independent of the concentration of the fluid used for the calibration, constantly turned out to be 1.4, VI may in the following be substituted by

$$\alpha_x = \alpha_k \cdot \frac{100 + p_x}{101.4} \dots \dots \dots \text{VII}$$

When for instance some body-fluid is measured against a standard containing 150 mM. NaCl/litre, the conductivity may often conveniently be expressed in NaCl-equivalents, that is in terms of the concentration of sodium chloride in water giving the same conductivity, SUNDERMAN (1926).

For this computation we may within certain limits make use of the following equation

$$x = 150 \cdot \frac{100 + p_x}{100 + p_0} \dots \dots \dots \text{VIII}$$

On the assumption that it is permissible to ignore the effects of any differences in electrolytic polarisation and temperature between the two vessels, equation VIII involves only two approximations:

1) The conductivity of the distilled water used for dilution is neglected.

2) The activity coefficient of the standard NaCl-solution is taken as equal to that of a NaCl-solution possessing the same conductivity as the unknown solution.

It is obvious that the errors introduced by these approximations are zero when $p_x = p_0$, and that they will increase with the difference between p_x and p_0 .

By increasing dilution of the fluids, previous to the measurement of the electrical conductivity, the above mentioned approximation 1) will cause an increasing error, whereas approximation 2) will cause a decreasing error.

To get an idea of the degree of dilution which we shall prefer for our measurements we will consider the following example.

We want to determine the conductivity x expressed in NaCl-equivalents of a solution of NaCl the concentration of which is exactly

90 per cent of the standard NaCl-solution applied, and we want to figure out the concentrations of salts in the electrode vessels causing an error, due to the approximations in question, which is just 0.2 per cent.

We will first consider the influence of the conductivity of the distilled water.

We call the specific conductivity of the distilled water used, κ_w and the conductivity caused by the NaCl of the sample to be measured and by the NaCl of the standard solution, κ_{xc} and κ_{kc} respectively.

We get the error in question:

$$\Delta = \frac{\kappa_x}{\kappa_k} - \frac{\kappa_{xc}}{\kappa_{kc}} \dots \dots \dots \text{IX}$$

$$\Delta = \frac{\kappa_{xc} + \kappa_w}{\kappa_{kc} + \kappa_w} - \frac{\kappa_{xc}}{\kappa_{kc}} \dots \dots \dots \text{X}$$

$$\Delta = \frac{\kappa_w (\kappa_{kc} - \kappa_{xc})}{\kappa_{kc} (\kappa_{kc} + \kappa_w)} \dots \dots \dots \text{XI}$$

We now put $\kappa_{xc} = 0.9 \kappa_{kc}$ and get

$$\Delta = \frac{\kappa_w \cdot 0.1 \cdot \kappa_{kc}}{\kappa_{kc} (\kappa_{kc} + \kappa_w)} \dots \dots \dots \text{XII}$$

it is seen that if we put $\kappa_{kc} = 50 \cdot \kappa_w$, Δ will be just 0.002.

As κ_w of the grade of distilled water applied by the author, was about $2 \cdot 10^{-6}$, κ_{kc} should be $\geq 50 \cdot 2 \cdot 10^{-6} = 100 \cdot 10^{-6}$ which corresponds to a NaCl-concentration of about 0.8 mM./litre.

Next we consider the influence of the activity coefficients in a pure solution of NaCl.

We assume that the activity coefficients obey the law of DEBYE & HÜCKEL which referring to monovalent ions may be written

$$-\log f = 0.50 \cdot \sqrt{m} \dots \dots \dots \text{XIII}$$

where m is the concentration in mols./litre. We call the activity coefficients of the solutions compared, f_x and f_k and get

$$-\log f_k = 0.50 \cdot \sqrt{m_k} \dots \dots \dots \text{XIV}$$

and

$$-\log f_x = 0.50 \cdot \sqrt{m_x} \dots \dots \dots \text{XV}$$

where m_k and m_x represent the concentration (M./litre) of the dilution to be figured out.

If, corresponding to an error of 0.2 per cent, we put $\frac{f_x}{f_k} = 1.002$ we have

$$\log f_x - \log f_k = \log 1.002 \dots \dots \dots \text{XVI}$$

Subtracting XV from XIV and inserting XVI we get

$$\log 1.002 = 0.50 \cdot (\sqrt{m_k} - \sqrt{m_x}) \dots \dots \dots \text{XVII}$$

inserting $m_x = 0.9 \cdot m_k$ we have

$$0.5 (\sqrt{m_k} - \sqrt{0.9 m_k}) = \log 1.002$$

$$\sqrt{m_k} = \frac{\log 1.002}{0.5 (1 - \sqrt{0.9})}$$

$$m_k = 0.0012 \text{ M./litre}$$

$$\text{or } m_k = 1.2 \text{ mM./litre.}$$

From these considerations it is seen that concerning measurements on NaCl (or similar salts of monovalent ions) the minimum total error involved in equation VIII, due to the approximations 1) and 2), page 5, will be obtained when the concentration of salts in the electrode vessels is about 1 mM./litre. If the concentration of salts in the solution to be measured is between 90 and 110 per cent of the concentration of the standard solution, the maximum error caused by each of the approximations in question will be smaller than ± 0.2 per cent.

It may easily be realised that the errors committed by neglecting the approximations 1) and 2) both are positive when the solution to be measured is less concentrated than the standard solution, whereas the errors are negative when the concentration of the solution to be measured exceeds that of the standard solution.

When polyvalent ions are present it may be desirable to carry out the measurement at a greater dilution to compensate for the influence of the greater ionic strength of these salts upon the activity coefficients.

In such circumstances it may be desirable to apply the correction for the conductivity of the distilled water used *e. g.* by means of equation XI page 6.

The apparatus described in this paper has been in use in this laboratory for several months and the possibility that temperature differences might influence the measurements have been watched. The cells are mounted close beside each other in a room facing north and protected against draught and unsymmetrical irradiation from the window, the heat radiator and the incandescent lamps in the neighbourhood. No measurable differences in the readings which could be ascribed to temperature differences have ever been noticed in these circumstances.

The electrodes of the vessels have been used uncoated as well as coated with platinum black (a rather thin layer). The capacities of resistance of the vessels before coating were 0.83 and 0.84 respectively and after coating 0.82 and 0.83.

If we want to be able to determine the equilibrium of the bridge with the maximum accuracy using uncoated electrodes, the concentration of salts in the fluid in the electrode vessel should not exceed about 2 and 50 mE./litre corresponding to the application

for the measurement of 50 and 1 000 Hz respectively. For coated electrodes the limits are about 50 and > 150 mE./litre corresponding to 50 and 1 000 Hz respectively.

By great care to avoid contamination of the fluids with carbon dioxide from expired air it was found possible to compare conductivities of pure NaCl-solutions down to $5 \cdot 10^{-6}$ ohm $^{-1}$ cm $^{-1}$ applying the correction for the conductivity of the distilled water used = $- \Delta$ (equation XI), with an error of about 2 per cent. κ of the distilled water used for this experiment was $1.3 \cdot 10^{-6}$.

Coated electrodes could be used for this experiment quite as well as uncoated. But coated electrodes which had been used for organic fluids as dilutions of blood serum turned out to be unusable for this purpose for months.

In order easily to be able to obtain the maximum accuracy stated the present method requires an amount of salts of at least about 10^{-6} mols corresponding to 1 ml of a solution containing about 1 mM./litre which again corresponds to a volume of about 7μ l of most biological fluids. Dealing with smaller quantities the error generally will increase.

The main cause of error when measuring very small samples of physiological saline solutions generally is the limited accuracy with which a constant delivery into the vessels could be obtained. For this reason it was not attempted to diminish the quantity of fluid required by reducing the dimensions of the electrode vessels.

The arrangement described has been used for determinations of the conductivity of a number of samples of human blood serum of about 10μ l carried out against the same amount of a NaCl-solution containing 150.0 m E./litre. Simultaneous determinations of the sum of kations, the "total base", was carried out by a modification of the electrodialytic method of KEYS (1936) described by the writer (HOLM-JENSEN 1943).

The results of these analyses were in close agreement with SUNDERMAN et al. (1926), who found the relation between total base, expressed in m E./litre, and conductivity in NaCl-equivalents to be given by the equation:

$$\text{total base} = 1.13 \cdot \text{conductivity.}$$

SUNDERMAN et al. carried out determinations of the conductivity on undiluted serum or plasma and corrected for the retarding effect of proteins, which was taken to be equal to 2.2 per cent

per each per cent protein present (GRAM & CULLEN 1923). The maximum difference between total base computed from the above equation and determined according to the method of STADIE & ROSS (1925) was in the material of SUNDERMAN 5 per cent; in 90 per cent of the determinations the difference did not exceed 3 per cent.

Applying the procedure, described in the present paper using samples of about 10 μ l, the correction for the retarding effect of protein need not be considered. However, it was often found convenient in routine work to use samples of 0.1 ml. of serum and of the standard NaCl-solution in each vessel. Under these circumstances an empirical correction of about 20 per cent should be added to the conductivity determined, before inserting in the above formula.

The writer has hitherto carried out about 25 simultaneous determinations of conductivity, concentration of kations and concentration of protein on samples of serum containing between 144 and 158 m E. kations/litre and between 5.0 and 9.0 per cent protein and has found the formula of SUNDERMAN to be valid with a maximum difference of ± 3 per cent. In 23 out of 25 determinations, the difference did not exceed 2 per cent.

As hitherto only samples of serum of fairly normal composition have been analysed by the writer, it cannot yet be established whether the better agreement found is due to a more uniform material of blood than that analysed by SUNDERMAN et al., or if it is caused by the elimination of errors connected with the application of the correction for the retarding effect of proteins upon conductivity.

The writer is at present preparing a study to be published shortly on the relation between the electrolytic conductivity and the concentrations of kations in serum and plasma from patients showing abnormal concentrations of proteins, chloride and bicarbonate.

Summary.

A simple procedure is described suitable for measurements of the electrolytic conductivity of small samples of saline solutions.

The samples are diluted, physiological solutions generally between 10 and about 100 times, and the electrolytic resistance is measured in proportion to the same degree of dilution of some

suitable standard solution placed in another electrode vessel, making use of a commercial Wheatstone-bridge arrangement.

The minimum quantity of physiological saline solutions required is of the order of 10 μ l.

It is possible without special precautions to carry out the estimations with an error not exceeding a fraction of one per cent. The factor generally limiting the accuracy obtainable is the accuracy with which exactly equal volumes of the two different solutions compared can be delivered.

The formula of SUNDERMAN et al.: total base = $1.13 \cdot \text{conductivity}$ is confirmed.

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On "Lipoid Solubility".

By

RUNAR COLLANDER.

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1. Introduction.

Almost half a century has already elapsed since OVERTON (1899) presented his much discussed lipoid theory of protoplasmic permeability. Yet it must be admitted that even now it is not possible to state exactly just how closely the penetration power of different solutes corresponds to their relative lipoid solubility, *i. e.*, to their distribution coefficients in the system plasma-membrane-lipoid/water. To solve this question it is, of course, equally necessary to know both the lipoid solubility of the permeating substances and their penetration power. It seems, however, that the interest of the investigators in this field has been so keenly focussed on the determination of the penetration power that the question of the lipoid solubility has been seriously neglected. It is true that the expression "the lipoid solubility" is found abundantly in the literature of protoplasmic permeability, but in nine cases out of ten only as a vague, loosely used phrase. In fact, we still possess very few exact data concerning the true lipoid solubility of different substances.

This is due not only to the troublesome fact that the chemical nature of the plasma membrane lipoids is not yet definitely known. Another obstacle is that the determination of the solvent properties of those substances — phosphatides and sterines — which are generally supposed to be the main components of the

plasma membrane is beset with particular difficulties: at room temperature they are solid substances which will swell in contact with water giving rise to colloidal systems of a complicated structure.

In cell physiology it has, therefore, become customary to use some other solvents — *e. g.* ethyl ether or olive oil — which are much easier to study as model substances for the unknown plasma membrane lipoids. This is, however, a rather precarious practice. An example! Butyramide and tricarballic acid have both the same relative ether solubility, viz., 1/17, but in the system olive oil/water the distribution coefficient of the former substance is 1/110, that of the latter about 1/10,000. Which of these figures indicates the true “lipoid solubility” of these substances? I think we must admit that our present knowledge does not suffice for the answering of that question. The least that must be demanded before such alleged lipoid solubility values are approved is that it has been made clear in what respects the solvent properties of the different organic solvents are alike and in what respects they differ. Only after that has been done will it be possible to evaluate with some degree of certainty the lipoid solubility of substances on the basis of their distribution in a more or less arbitrarily chosen “model system”.

The importance of such studies was already stressed by OVERTON (1902 p. 259) who himself carried out some important pioneer work in this direction (OVERTON 1899, 1902, 1907). However, since then this field has been badly neglected by physiologists as well as by students of physical chemistry. In fact there is, at least as far as the present writer knows, no comprehensive survey of the distribution of different solutes between organic solvents and water which would essentially surpass the one given by OVERTON forty years ago. Also the compilations of distribution coefficient values given *e. g.* in the *International Critical Tables* or in the handbook of LANDOLT-BÖRNSTEIN are rather unsatisfactory from the standpoint of the cell physiologist. Thus, they include scarcely any data referring to the distribution of such physiologically important substances as the polyvalent alcohols, the sugars, the amino acids etc.

The overwhelming majority of the distribution determinations hitherto published refers to the distribution of organic acids. The papers of SMITH (1921, 1922), SMITH and WHITE (1929), BODANSKY (1929), BODANSKY and MEIGS (1932), TAYLOR (1930), KOLOSSOWSKY,

KULIKOW and BEKTUROW (1935), ENGLAND and COHN (1935) (amino-acids) and DERMER, MARKHAM and TRIMBLE (1941) are perhaps the most important modern contributions of this kind. The distribution of inorganic acids has been studied chiefly by ARCHIBALD (1932), that of amines and other bases especially by SMITH (1921) and POIJÄRVI (1928), that of dyes chiefly by NIRENSTEIN (1920), HÖBER and PUPILLI (1933) and RUGE (1940), that of non-electrolytes by BÄRLUND (1929), COLLANDER and BÄRLUND (1933) and MEYER and HEMMI (1935). Among recent contributions to the general theory of the distribution of organic solutes the papers of FRUMKIN (1925) and of MEYER and HEMMI are the most important.

In the hope of filling at least some of the regrettable gaps in our knowledge of the distribution phenomena, with special attention to the problem of lipoid solubility, the present writer has started an investigation into the distribution of various solutes between water and several organic solvents. Some general results so far arrived at are presented here as quite preliminary in character. The chief aim has been to get a general idea of the similarities and differences between different organic liquids as to their solvent properties. In subsequent papers it is hoped to clarify this matter more in detail, and also to elucidate the correlations between the molecular structure of different solutes and their distribution.

My enquiry is based chiefly on distribution coefficient values determined especially for this purpose and mostly by Mr. VÄINÖ HEIKINHEIMO to whom I am deeply indebted for his careful work.

It should, however, be pointed out that no great accuracy is claimed for the distribution coefficient values presented here; it has been thought wiser to make, at least to begin with, a general survey of a wider field than to determine single distribution coefficients with the greatest possible degree of precision. Especially the smallest distribution coefficient values are, owing to technical difficulties, rather inaccurate. (Some of the values used here are as small as about $1/10,000$ while the values to be found *e. g.* in the *International Critical Tables* are seldom below $1/100$.) Most solutes have been studied in one or two concentrations only. (The more concentrated phase generally contained about 0.1 to 1 mol/litre.) The experiments were carried out at a temperature varying between about 16° and 22° C. (Fortunately the distribution is only slightly influenced by temperature.) The dissociation of the acids and bases has not been taken into account in calculating the distribution coefficients nor has the molecular association occurring especially in the non-aqueous phase. All the distribution coefficient values given here are thus only "gross distribution ratios".

2. Comparison of Various Organic Solvents.

Fig. 1 shows the distribution coefficients of some twenty organic acids in the system iso-butyl alcohol/water, sec. octyl alcohol/water, and oleic alcohol/water plotted against their distri-

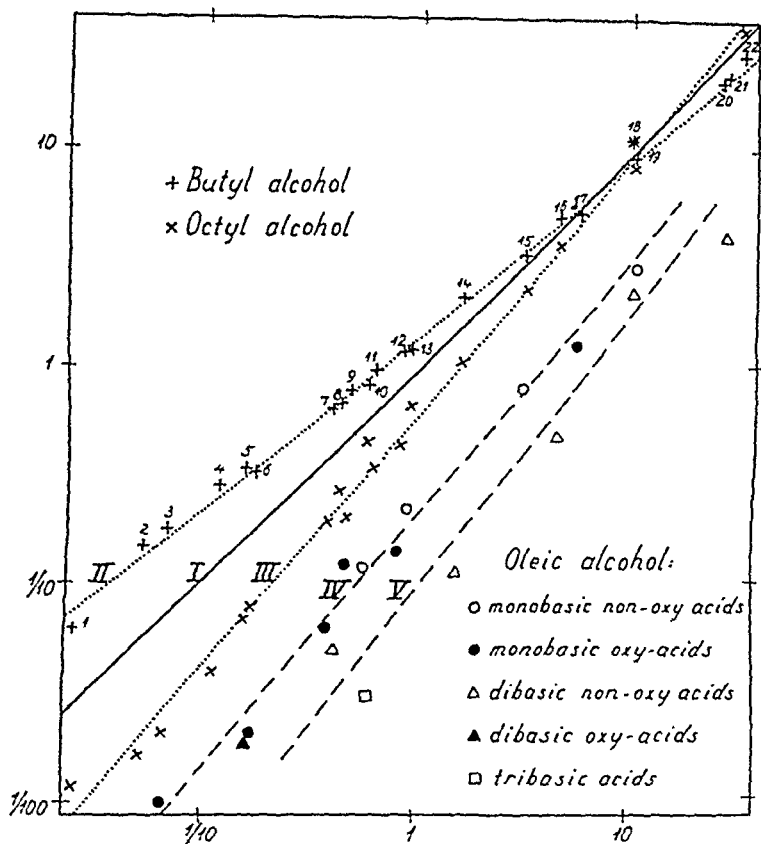


Fig. 1. Abscissas: distribution coefficients iso-amyl alcohol/water. Ordinates: distribution coefficients iso-butyl alcohol/water, sec. octyl alcohol/water, and oleic alcohol/water. Solutes: 1 gluconic, 2 tartaric, 3 glyceric, 4 citric, 5 malic, 6 glycollic, 7 lactic, 8 malonic, 9 pyruvic, 10 formic, 11 tricarballic, 12 oxy-iso-butyric, 13 acetic, 14 glutaric, 15 propionic, 16 dimethyl-malonic, 17 mandelic, 18 diethyl-malonic, 19 butyric, 20 valerianic, 21 benzyl-malonic, 22 phenyl-acetic acid. The points without figures refer to the same acids as the points exactly above them.

bution coefficients in the system iso-amyl alcohol/water.¹ The last-mentioned system is thus chosen as a kind of "standard" with which the others are compared. If the distribution in all the sys-

¹ These expressions are, of course, not quite correct. Instead of, for example, iso-butyl alcohol/water we ought really to write: (a saturated solution of water in iso-butyl alcohol)/(a saturated solution of iso-butyl alcohol in water). The expressions used above may nevertheless be allowed for the sake of brevity.

tems studied were the same, all points would lie on the straight line I, the angle of which with the horizontal is 45° . In reality they lie, however, for the most part either above or below this line.

Let k_{but} , k_{am} , k_{oct} , and k_{oi} denote the distribution coefficients in the systems iso-butyl alcohol/water, iso-amyl alcohol/water etc. Fig. 1 shows that the values of k_{but} lie rather regularly along the straight line II, the angle of which with the horizontal is distinctly smaller than 45° and which cuts the line I in a point which corresponds to a distribution coefficient value of about 10. Similarly the values of k_{oct} are grouped along the straight line III, the inclination of which is, however, distinctly greater than that of the line I. The lines I and III cut each other at about the same point as the lines I and II.

These results are easily understood, at least in principle, if we suppose that the distribution of the acids between alcohols and water is in the first place determined by the polarity of the solutes on the one hand and that of the solvents on the other in such a manner that each solute is most soluble in that solvent with which it agrees most closely as to its degree of polarity.¹ Thus, the acids 1—11 are so highly polar (hydrophilic) that their solubility in water surpasses their solubility in all the other solvents studied. The acids 12—18 agree most closely with the iso-butyl alcohol as to their degree of polarity and are therefore most soluble in this solvent ($k_{but} > k_{am} > k_{oct}$). The acids 19—21 seem to be most soluble in iso-amyl alcohol ($k_{but} < k_{am} > k_{oct}$). Finally the phenyl acetic acid (solute 22) is, of the acids studied, the only one which is so nonpolar (hydrophobic) in character that it is more soluble in sec. octyl alcohol than in any of the solvents so far mentioned ($k_{but} < k_{am} < k_{oct}$).

The fact that the points referring to the butyl and octyl alcohols lie so closely along the lines II and III is probably due to the great similarity between these solvents on the one hand and the "standard" solvent (amyl alcohol) on the other. That this is so is indicated by the fact that the oleic alcohol, which differs somewhat more from the standard, behaves in another manner than the solvents hitherto considered: the points referring to the oleic alcohol do not lie along one, but along at least two different lines, viz., the monobasic acids along the line IV, the di- and tribasic acids along the line V.

¹ Instead of the term polarity we could in this connection use the terms hydrophilia, lipophobia, or organophobia. The best measure of this quality is probably the dielectric constant.

The differences in the inclination of the lines are also easily understood, for the less the organic solvent differs from water as to its solvent capacity, the less will the distribution coefficients of the different solutes differ. (In the system water/water the "distribution coefficients" of all solutes would, of course, have the same value, viz., 1.) The fact that the inclination of the lines IV and V seems to be about the same as that of the line III might be due to an accident, for the points determining the lines IV and V are relatively few.

The most important conclusion to be drawn from Fig. 1 is that there exists an obvious correlation between the distribution coefficients in the different systems studied. Thus, if $k_{standard}$ denotes the distribution coefficient value of a certain solute in the standard system and k_x its value in another system, then the following equation is valid:

$$\log k_x = a \cdot \log k_{standard} + b.$$

In this equation a and b are two constants characterizing the solvents in question. In certain cases they are, however, also to some extent influenced by the nature of the solutes. Their value can be calculated from data shown in Fig. 1.

Thus, for example, in the case of the system butyl alcohol/water as compared with the standard system amyl alcohol/water it can be seen from Fig. 1 that

$$\log 0.22 = a \cdot \log 0.1 + b \text{ and}$$

$$\log 9.6 = a \cdot \log 10 + b.$$

From this it may be computed that in this case $a = 0.82$ and $b = 0.16$. Therefore

$$\log k_{but} = 0.82 \cdot \log k_{am} + 0.16.$$

In just the same way it is found that

$$\log k_{oct} = 1.19 \cdot \log k_{am} - 0.21.$$

In order to calculate the distribution coefficients in the system oleic alcohol/water two different values for both a and b are needed, according to the nature of the acids studied. Thus, in the case of monobasic acids we have the equation

$$\log k_{ol} = 1.2 \cdot \log k_{am} - 0.70$$

and in the case of di- or tribasic acids

$$\log k_{ol} = 1.3 \cdot \log k_{am} - 1.04.$$

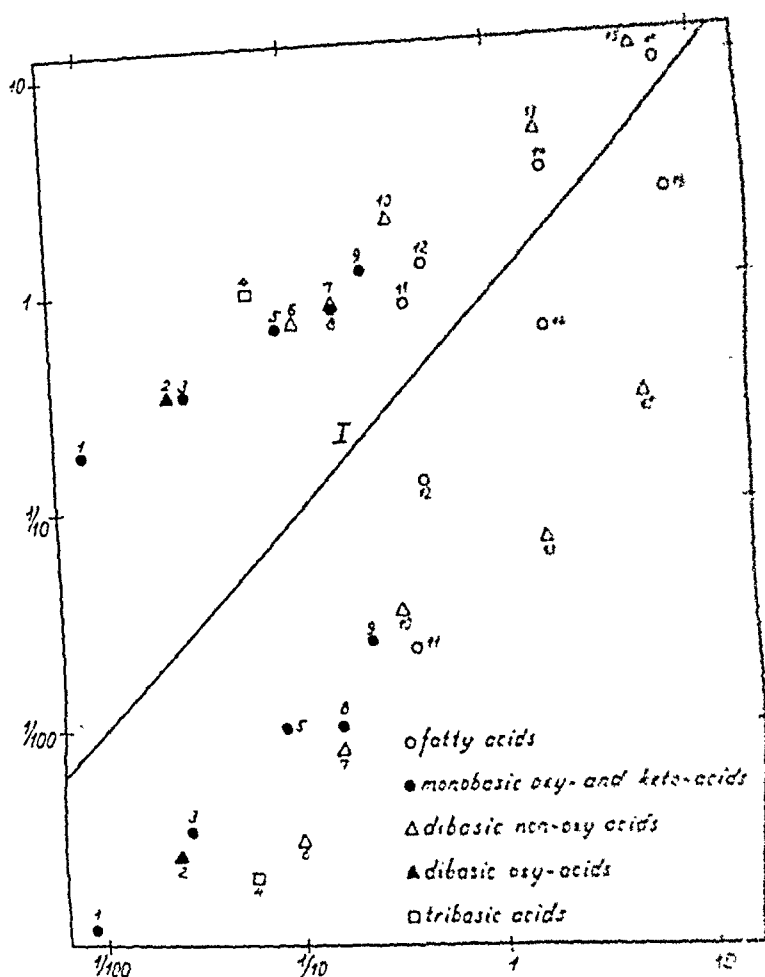


Fig. 2. The distribution coefficients of various organic acids in the systems iso-butyl alcohol/water (points above the inclined line) and oleic acid/water (points below that line) plotted against the distribution coefficients of these acids in the system ethyl ether/water. 1 glyceric, 2 malic, 3 glycollic, 4 tricarballic, 5 lactic, 6 malonic, 7 maleinic, 8 pyruvic, 9 oxy-iso-butyric, 10 glutaric, 11 formic, 12 acetic, 13 dimethyl-malonic, 14 propionic, 15 diethyl-malonic, 16 butyric acid.

The constant a is evidently an expression for the inclination of the straight lines on which the single points lie or, in other words, an expression for the differences between the distribution coefficient values of the solutes, for if $a < 1$, then these differences are smaller than in the standard system, while, if $a > 1$, they are greater than in this system. At the same time a may serve as an indication of the polarity of the organic solvent considered, for the greater a is, the more non-polar, *ceteris paribus*, is the solvent in question.

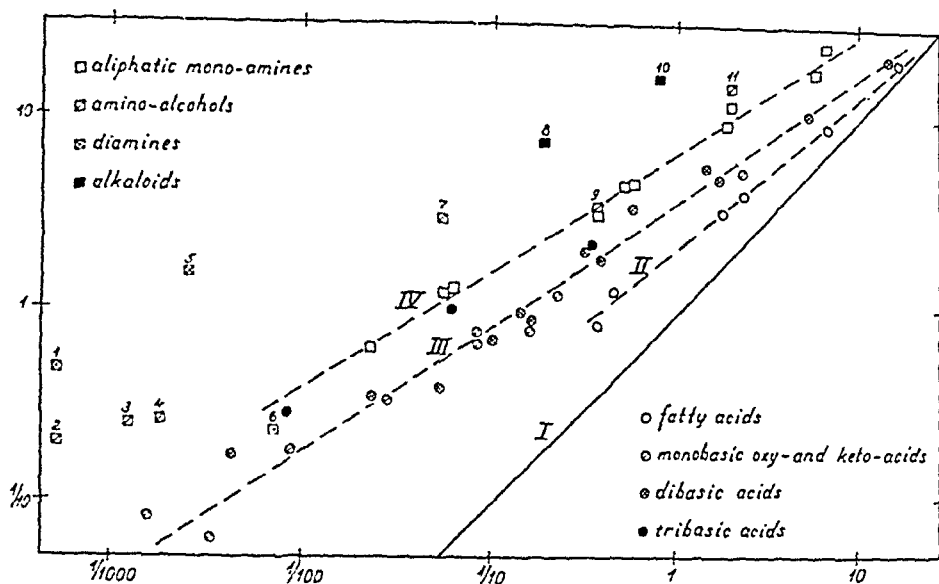


Fig. 3. Abscissas represent distribution coefficients iso-butyl alcohol/water, ordinates distribution coefficients ethyl ether/water. The most outstanding points are: 1 tetramethylene-diamine, 2 diethanol-amine, 3 ethanol-amine, 4 triethanol-amine, 5 pentamethylene-diamine, 6 ammonia, 7 tropin, 8 morphine, 9 diethyl-ethanol-amine, 10 codeine, 11 ephedrine.

The constant b , on the other hand, is an expression for the position of the oblique lines on the ordinate. Thus, if we choose a solute the k_{standard} of which = 1, then b gives directly the distribution coefficient of this solute in the other systems.

From Fig. 2 it can be seen that such empirical rules do not apply only to solvent systems composed of various alcohols and water but also when we compare systems of which the organic phases are rather different in nature: an alcohol, an ether, and an acid. In studying Fig. 2 one is struck by the fact that the points referring to butyl alcohol on the one hand and oleic acid on the other occupy distinctly symmetrical positions in relation to each other. In other words: the more the distribution coefficient value of a solute in the system butyl alcohol/water differs from its value in the system ether/water, the more also does its value in the system oleic acid/water differ from its value in that system. On p. 378 we will return to this fact.

The empirical rules so far mentioned do not apply only to the distribution of acids but also to the distribution of weak bases. That is seen in Fig. 3 which shows the distribution of organic acids and bases in the systems iso-butyl alcohol/water and ethyl

ether/water. The straight line I indicates the position of the points if the distribution coefficient values were the same in both systems. In reality, however, all the solutes here considered have greater distribution coefficient values in the system butyl alcohol/water than in the other, but the difference varies considerably according to the nature of the solute. Thus it is seen that the fatty acids lie all very regularly along the line II, oxy-acids and keto-acids, and also the dibasic acids along the line III, while the points representing the tribasic acids (citric, tricarballic, and aconitic acid) lie somewhat higher. Still higher lie the points of the aliphatic mono-amines situated along the line IV. Also ammonia (point 6) joins this group. The highest positions are occupied by the diamines, the amino-alcohols (only diethyl-ethanol-amine is an exception; it seems to join the alkylamines), and the alkaloids. Because of the great number of solutes included in Fig. 3 — they are about 50 in all — only those which occupy a somewhat outstanding position are marked by figures.

Fig. 4 refers to two solvents, viz., ethyl ether and olive oil, which being easily accessible have been used rather frequently as a kind of model for the plasma membrane lipoids. The solutes included in this graph are in part weak acids, but in part also non-electrolytes. All of them are less soluble in olive oil than in ether. There are, however, also in this case distinct differences in the behaviour of different kinds of solutes. Thus, the amides studied are rather constantly 2—4 times less soluble in oil than in ether. The line II along which the amides are grouped runs therefore rather parallel with the basic line I the inclination of which is 45° . This might, however, be due partly to accidental errors in the determination of the distribution coefficients and, especially, to the fact that the amides studied happen all to be strongly polar, while more hydrophobic ones would probably be even more soluble in olive oil than in ether. The monobasic acids lie rather regularly along the line III. They thus follow the equation

$$\log k_{oil} = a \cdot \log k_{ether} + b,$$

in which $a = 1.47$ and $b = -1.07$. From that it may be computed that the lines I and III would cut each other at a point corresponding to a distribution coefficient of about 200, which seems rather plausible. The di- and tribasic acids comprised in Fig. 4 are so few in number that it would be difficult to draw a line representing them. The nitrogen-free non-electrolytes (alcohols, ether-alcohols,

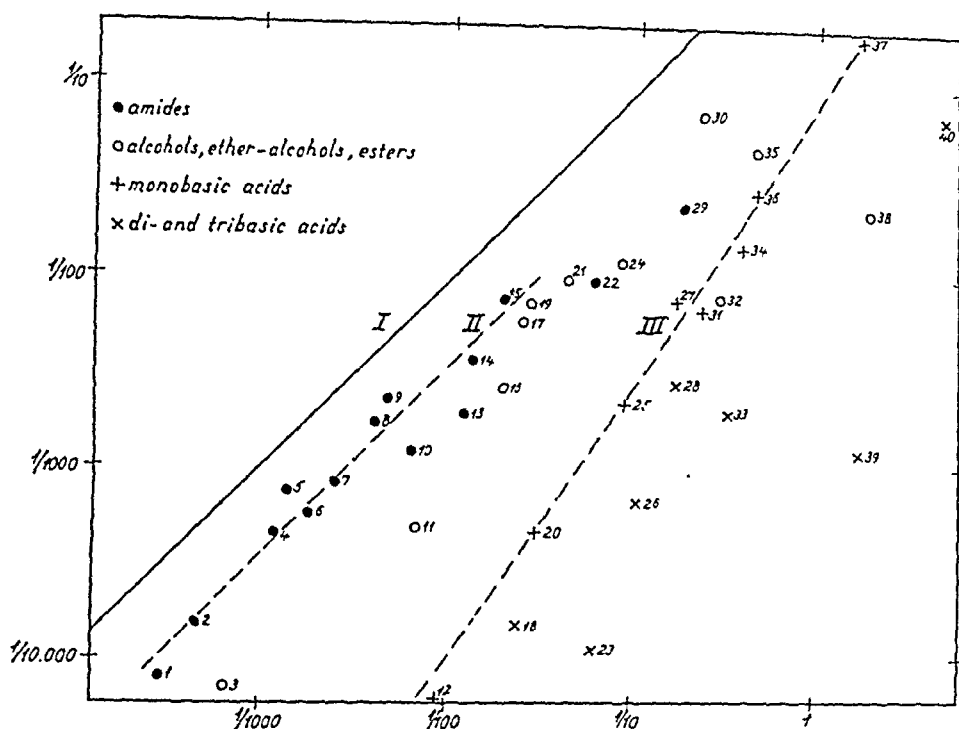


Fig. 4. Abscissas: distribution coefficients ethyl ether/water. Ordinates: distribution coefficients olive oil/water. Solutes: 1 malonamide, 2 urea, 3 glycerin, 4 methyl-urea, 5 formamide, 6 lactamide, 7 acetamide, 8 ethyl-urea, 9 dimethyl-urea, 10 thiourea, 11 glycol, 12 glyceric acid, 13 diethyl-malonamide, 14 propionamide, 15 diethyl-urea, 16 glycerin-methyl-ether, 17 propylene glycol, 18 malic acid, 19 glycerin ethyl-ether, 20 glycollic acid, 21 mono-acetin, 22 butyramide, 23 tricarballylic acid, 24 mono-chlorhydrin, 25 lactic acid, 26 malonic acid, 27 pyruvic acid, 28 maleinic acid, 29 valeramide, 30 diacetin, 31 oxy-iso-butyric acid, 32 methyl alcohol, 33 glutaric acid, 34 formic acid, 35 trimethyl-citrate, 36 acetic acid, 37 propionic acid, 38 ethyl alcohol, 39 dimethyl-malonic acid, 40 diethyl-malonic acid.

and esters) are rather irregularly scattered between the lines II and III. The points referring to methyl and ethyl alcohol lie surprisingly far to the right; later investigations must show whether this result is correct or, perhaps, due to experimental errors.

While in the graphs hitherto presented only a few solvents have been compared, Fig. 5 allows the comparison of seven different solvents. The solutes, in this case ten organic acids, are arranged according to decreasing distribution coefficient values in the system ether/water. The acids have been chosen in such a way that every second acid is a monobasic one and the others di- or tribasic.

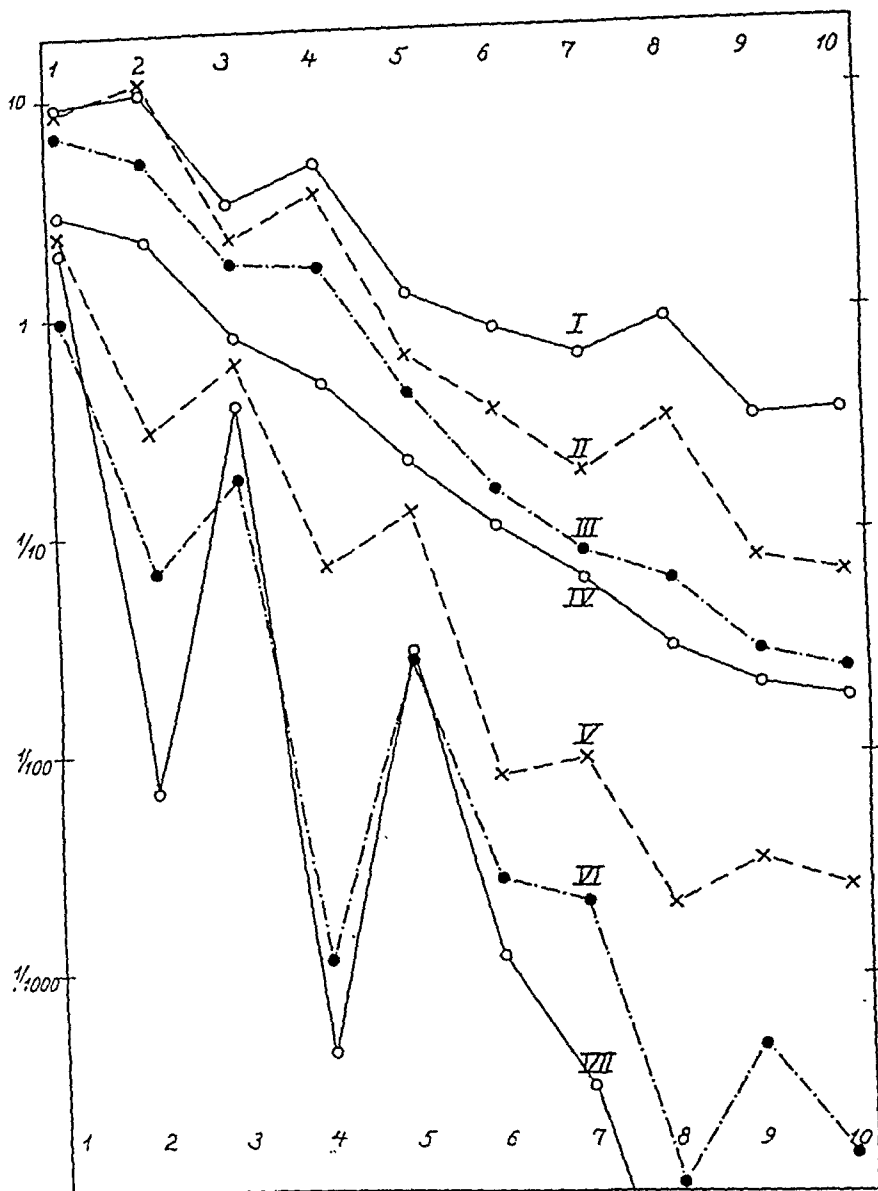


Fig. 5. The distribution of 10 organic acids between different organic solvents and water. Solutes: 1 butyric, 2 diethyl-malonic, 3 propionic, 4 dimethyl-malonic, 5 acetic, 6 malenic, 7 lactic, 8 tricarballic, 9 glycollic, 10 malic acid. Solvents: I iso-butyl alcohol, II sec. octyl alcohol, III ethyl ether, IV oleic alcohol, V oleic acid, VI olive oil, VII benzene. The ordinates represent distribution coefficients.

A study of Fig. 5 shows that all our solvents can be arranged in a series in which the dissolving power for all distinctly polar (hydrophilous) acids decreases regularly in the following sequence: iso-butyl alcohol > octyl alcohol > ethyl ether > oleic alco-

hol > oleic acid > olive oil > benzene. This result confirms the idea stated already on p. 367 that the distribution of organic solutes between different organic solvents and water is determined chiefly by the polarity of both solutes and solvents, each solute being most soluble in that solvent with which it agrees most closely in its degree of polarity. Just in this sense the old rule *similia similibus solvuntur* still holds good although there are numerous other cases in which it is not applicable (cfr. HILDEBRAND 1936 p. 14).

The curves of Fig. 5 converge towards the left and diverge towards the right. This is due to the fact that the more pronouncedly polar (or non-polar) a solute is, the greater are the differences between its distribution coefficient values in different systems, while solutes which are neither strictly polar nor strictly non-polar are more similarly distributed between the two phases of various systems.

A curious phenomenon, the reason of which is not yet clear, is that the curves do not spread evenly towards the right but so to say rhythmically: in passing from a monobasic to the following di- or tribasic acid a strong divergence is observed, while in passing from a di- or tribasic acid to the next monobasic one (the greater hydrophilia of which is due either to a greater number of hydroxyl groups or to a shorter carbon chain) there is a "contraction" of the curve system. The diverging of the curves occurs therefore much more evenly if only monobasic acids are considered as in Fig. 6, or only di- and tribasic.

The principal result of all the experiments described in this chapter is the conclusion that the solvent properties of all the solvents studied depend quite overwhelmingly on their varying degree of polarity, while other more specific solvent peculiarities seem to play only a minor rôle. Thus, among the solvents studied no especially good solvents for, say, fatty acids, or oxy-acids, or polybasic acids, have been found or, in fact, for any single kind of solutes, except for more or less polar compounds on the one hand, and for non-polar on the other. It thus seems possible, at least in principle, to express the solvent properties of all these solvents as functions of their degree of polarity — about in the same manner as the properties of the different kinds of light may be expressed as functions of their wave-length.

While we are compelled to regard polarity as the most important quality determining the solvent properties of different solvents, it would clearly be one-sided, if it was assumed that all

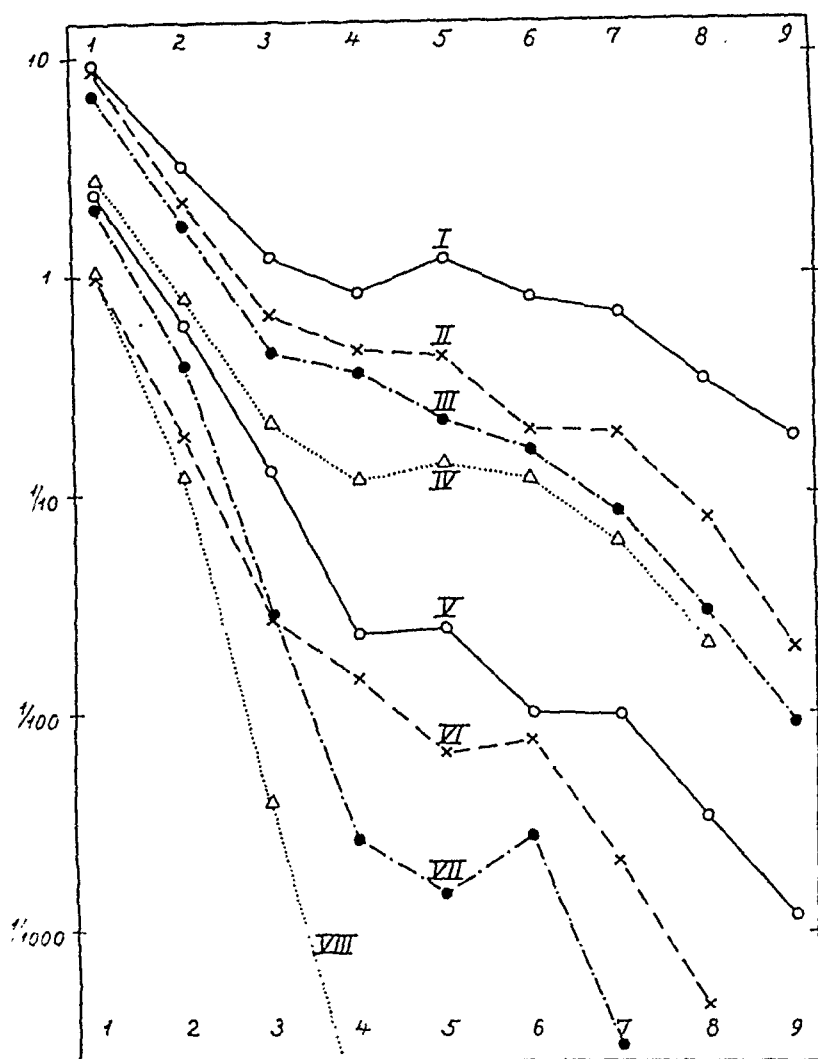


Fig. 6. The distribution of nine monobasic acids between different organic solvents and water. Solutes: 1 butyric, 2 propionic, 3 acetic, 4 oxy-iso-butyric, 5 formic, 6 pyruvic, 7 lactic, 8 glycollic, 9 glyceric acid. Solvents: I iso-butyl alcohol, II sec. octyl alcohol, III ethyl ether, IV oleic alcohol, V oleic acid, VI olive oil, VII benzene, VIII hexane. Ordinates represent distribution coefficients.

the differences in the solvent properties of organic liquids are due solely to polarity differences, There is, in fact, at least one other factor which in some cases influences the solvent power very strikingly. This is the acidity or basicity of the solvent: acid solvents have a special affinity for basic solutes while basic solvents have a similar affinity for acid solutes. This fact has been pointed out and studied to some extent by NIRENSTEIN (1920), HÖBER and PURILLI (1931), COLLANDER and BÄRLUND (1933), and others.

Probably also in some other cases certain kinds of reversible chemical reactions may occur between solvent and solute, thus enabling the solvent to take up more of certain solutes than would be expected when considering only the polarity of the substances in question. It seems, however, at least within the experience here recorded, that conspicuous examples of this are rare, so that they do not very materially influence the general picture. Further investigations are, however, needed to determine the range of such factors.

3. The Solvent Properties of the Plasma Membrane Lipoids.

If the views expressed in the preceding chapter are sound, the problem of the solvent properties of the plasma membrane lipoids can be virtually reduced to two main factors, viz., (1) their polarity, and (2) their acidity or basicity. Let us now consider how these two factors may be determined.

(1) *The polarity of the plasma membrane lipoids.* One way to get an idea of the polarity of the plasma membrane lipoids is to start from the probable chemical composition of the plasma membrane. This approach has been attempted by MEYER and HEMMI (1935). They suppose that the plasma membrane is composed mainly of sterines containing one hydroxyl group to about 18 carbon atoms. For this reason they propose oleic alcohol ($C_{18}H_{33}OH$) as a suitable model substance for the plasma membrane lipoids.

One objection to this proposition is that besides sterines, phosphatides, fatty acids, and neutral fats might also be constituents of the plasma membrane and that, as is seen from Figs. 5 and 6, at least the fatty acids of the type of oleic acid and the neutral fats of the type of olive oil are distinctly less polar (less hydrophilic) substances than oleic alcohol. But besides this yet another circumstance must be taken into account. The plasma membrane consists at least partially of regularly orientated molecules of which the hydrocarbon chains form distinct layers built up solely of these parallel chains. It seems, therefore, probable that even if the *mean* degree of hydrophilia in the plasma membrane is that of, say, oleic alcohol, nevertheless certain layers of it, namely those composed of the parallel hydrocarbon chains will be much more hydrophobic in nature, in fact probably about as hydro-

phobic as a hydrocarbon. If this is so, it is clear that it is just these layers which will chiefly control the passage of hydrophilic solutes through the plasma membrane.

In the light of these considerations it seems rather risky to base speculations as to the polarity of the plasma membrane on its supposed chemical composition. At any rate a more direct approach to the problem is very desirable and seems, in fact, not impossible. Let us suppose that the solute *A* penetrates the plasma membrane *n* times faster than another solute *B*. Under certain conditions (*e. g.* equal molecular size of *A* and *B*) this probably means that the relative solubility of *A* in the least permeable layer of the plasma membrane is about *n* times greater than that of *B*. On the other hand it is seen from Figs. 5 and 6 that the differences between the relative solubilities of different solutes in a certain organic solvent are the greater the more non-polar this solvent is. This fact can be illustrated by the following rather arbitrarily chosen example. If we examine (*cf.* Fig. 5) how many times greater the distribution coefficient of propionic acid is than that of malonic acid in systems composed of different organic solvents and water, we shall find that the ratio $k_{\text{propionic acid}} : k_{\text{malonic acid}}$ has the following values:

in	butyl alcohol water	octyl alcohol water	oleic alcohol water	ethyl ether water	oleic acid water	olive oil water	benzene water
	4.7	8.5	16	17	200	280	3000

If it is possible to decide, by means of permeability experiments, how many times the distribution coefficient plasma-membrane-lipoid/water for the solute *A* (say, propionic acid) is greater than that of another solute *B* (say, malonic acid), then it should be possible to estimate the polarity of the plasma membrane (more exactly: of that plasma membrane layer which offers the chief resistance to the passage of these solutes) as compared with the polarity of given organic solutes.

A preliminary attempt to estimate the polarity of the plasma membrane of *Chara* along such lines was made by COLLANDER and BÄRLUND (1933 p. 95). They found that the polarity of the plasma membrane substance agreed roughly with that of olive oil. This result was, however, admittedly only a very crude one. It is, therefore, important to achieve more accurate determinations of this sort. Probably it will be found that the degree or

polarity varies, at least in some cases, markedly from one kind of cell to another. (Cfr. ELO 1937 p. 101.)

There is still, however, a practical difficulty to be overcome before the distribution coefficient values in such a system can be determined. The solubility of many physiologically important substances (*e. g.* sugars, polyvalent alcohols, amino acids, inorganic salts etc.) in lipid-like organic solvents is so extremely low that the distribution coefficient values are of the magnitude of only 1/100,000 or perhaps even 1/1,000,000. The direct determination of such values is, of course, very difficult, yet there is an indirect method which might, perhaps, be useful in such cases. The principle involved can be explained on the basis of Fig. 2. A study of this graph shows that if a point indicating the distribution butyl alcohol/water lies, say, n units above the line I which represents its distribution ether/water, then the point indicating its oleic acid/water distribution lies on an average about $1.5 n$ units below that line. It is thus possible to compute approximately the distribution coefficients of different solutes in the system oleic acid/water on the basis of the considerably greater and therefore more easily determinable distribution coefficients of these solutes in the systems butyl alcohol/water and ether/water. The equation for this is

$$k_{oleic\ acid} = k_{ether} : \frac{1.5\ k_{butyl\ alcohol}}{k_{ether}} = \frac{(k_{ether})^2}{1.5\ k_{butyl\ alcohol}}.$$

Table I shows that the values calculated in this way agree tolerably well with those experimentally determined. (The most deviating values are given in parentheses.) It should also be noted that the deviations found are not due solely to faults in the calculation method, but probably also largely, perhaps even mostly, to purely experimental errors.

(2) *The acidity or basicity of the plasma membrane lipids.* It has been pointed out by WILBRANDT (1931), COLLANDER and BÄRLUND (1933), MARKLUND (1936), ELO (1937) and others that there are clear indications of a varying acidity or basicity of the plasma membrane lipids of different kinds of cells. These differences can be detected by comparing the permeability of the protoplasts to solutes of about equal polarity and equal molecular size, but differing as to their basicity or acidity. The conclusions hitherto drawn from experiments of this kind are mainly qualitative in character. It will, however, probably not be impossible to

work out methods for more quantitative determinations of the acidity or basicity of the plasma membrane lipoids.

Table I.

	k_{but}	k_{ether}	$k_{\text{oleic acid}}$ calculated experiment	
Butyric acid	9.4	6.8	3.3	2.5
Propionic acid	3.3	1.7	0.62	0.62
Diethyl-malonic acid	11	5.2	(1.7)	0.30
Acetic acid	1.2	0.44	0.10	0.13
Dimethyl-malonic acid	1.7	3.0	(0.37)	0.070
Glutaric acid	2.0	0.31	0.031	0.034
Oxy-iso-butyric acid	1.2	0.22	0.027	0.025
Formic acid	0.80	0.16	(0.11)	0.023
Pyruvic acid	0.80	0.16	0.021	0.099
Lactic acid	0.68	0.084	0.0070	0.0098
Maleinic acid	0.88	0.16	0.019	0.0071
Glycolic acid	0.33	0.028	0.0016	0.0033
Malonic acid	0.69	0.10	0.0090	0.0031
Malic acid	0.35	0.024	0.0011	0.0026
Tricarballic acid	0.97	0.060	0.0025	0.0021
Glyceric acid	0.18	0.0090	(0.00029)	0.0012

Summary.

In order to evaluate the distribution of solutes in the system plasma-membrane-lipoid/water on the basis of their distribution in more or less arbitrarily chosen "model systems" such as ethyl ether/water or olive oil/water it seems necessary to know in what respects the solvent properties of different organic solvents differ from each other and what they have in common. The present paper gives a preliminary account of studies which aim at elucidating these questions.

The results so far obtained indicate that it is possible to reduce the solvent properties of organic solvents to two major factors, viz., (1) their polarity (hydrophilia), and (2) their acidity or basicity, while other, more specific properties, seem almost negligible compared with these. When the second factor is not involved, the polarity of the solvent is, thus, by far the most important. In such cases the different solvents can be arranged (as in Figs. 5—6) in a continuous series or scale in which the solvent properties of each solvent are determined by its position on the scale.

If the distribution coefficients of given organic solutes in the system solvent I/water are plotted against the distribution coef-

ficients of the same solutes in another system, viz., solvent II/water, using a logarithmic scale, then the points denoting the distribution coefficients will be found lying either along one straight line or — when the solvents differ more — along several straight lines, each line referring to a given homologous series (or a group of similar homologous series) of solutes (Figs. 1—4). Thus, if k_I and k_{II} denote the distribution coefficients in the two systems, we have the equation

$$\log k_I = a \cdot \log k_{II} + b$$

where a and b are two empirical constants.

As a further example of the correlations found it may be mentioned that the distribution coefficients of hydrophilic organic acids in the system oleic acid/water can be approximately calculated from their distribution coefficients butyl alcohol/water and ethyl ether/water according to the empirical equation

$$k_{oleic\ acid} = \frac{(k_{ether})^2}{1.5\ k_{butyl\ alcohol}}$$

Also in the case of the unknown lipoids of the plasma membrane it seems most important to determine their polarity and their acidity or basicity. Methods for pursuing this are discussed.

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An Experimental Study of the
Influence of Haemorrhagic Anaemia
on the Ovarian Function

By

GÖSTA SUNDELIN

STOCKHOLM 1946

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Chapter I.

Introduction.

Chlorosis is the disease that first aroused interest in the correlation anaemia—ovarian disturbances. When chlorosis had its heydays at the end of the nineteenth century, the general opinion was no doubt that this disease was caused by changes in the ovaries. VIRCHOW, V. NOORDEN, JAGIC, DIEPGEN and SCHRÖDER, and later also NAEGELI and others, supported that opinion. Thanks to the works of MERKIN, HEILMEYER, WALDENSTRÖM and SCHULTEN and others we now know, however, that conditions rather are reversed, *i. e.* that the anaemia is the primary factor and the ovarian disturbances secondary to the anaemia.

The ample literature on hypochromic anaemia contains reports from some researchers, such as MEULENGRACHT, GRAY and WINTROBE, HILDEBRANDT, MEYERS and co-workers, FOWLER, BARER and others, to the effect that they have found no disturbances in the genital sphere as a result of anaemia, but that anomalies in the genital region contribute to the anaemia. Other scientists, KAZNELSON, DAMESHEK, HADEN and SINGLETON, SCHUR, WILKINSON, LUNDHOLM and others found decided changes above all in the menstruation in cases of hypochromic anaemia.

Interesting experimental studies of the ovarian function as influenced by undernourishment have been carried out, *inter alia*, by SCHMIDT, who had to diet several generations of rats on food poor in Fe in order to produce true anaemia in the test animals. The generative ability began to fail in the third generation; the fourth generation, if it appeared at all, consisted of small, poorly developed animals, which, however, became normal after a few weeks' treatment with iron.

The literature quoted has not made a detailed study of the influence of anaemia on the genital sphere. On the initiative of Professor AXEL WESTMAN the present paper was begun with a view to contributing to the solution of this problem. The investigations thus being of a pioneer character, it was considered necessary to attack the problem experimentally.

Chapter II.

Test Animals.

The rat was selected as a suitable test animal, it being well known that that animal has a regular sexual cycle, the phases of which can easily be checked by vaginal smears. Data on the blood morphology, sexual biology, etc., of the rat have been taken from DONALDSON and from LONG and EVANS.

The various stages in the ovarian cycle of the rat are illustrated in Table 1. A study of this table discloses that stages 2 and 3 may very well be made into one and that we thus only need consider four stages, *viz.* pro-oestrus, oestrus, metoestrus and dioestrus, abbreviated pro, pos, met, and neg, which abbreviations will be used in the following to denote the various stages. By determining the prevailing stage every morning, it is possible to obtain a picture of the ovarian function sufficiently exact for the present work. In the following met and neg are looked upon as expressions for a lacking ovarian impulse and are therefore placed on the same level in the diagrams.

The vaginal cycle ceases 1) during pregnancy, 2) during pseudo-pregnancy, 3) at the end of the fertile period. The latter occurs in the rat at the age of 15—18 months. Among these “negative” stages of function, it is only pseudo-pregnancy that may be difficult to avoid in the test animals. It may, as is well known, arise in many ways, for instance by the cervix being touched by the spatula used for taking vaginal smears. This can be avoided, however, by not inserting the spatula into the upper part of the vagina.

The blood volume of the rat varies according to the body weight. According to DONALDSON the female rat has the following quantity of blood:

at a weight of 100 g	6—7 ml
» » » » 150 g	9 »
» » » » 200 g	13 » , etc.

Table 1.

Stages in the ovarian cycle of the rat.
(Modified table according to Long and Evans).

Stage	Vaginal smear	Uterus	Ovary	Heat
I about 12 hrs	Nuclear epithelial cells only	Collection of fluid in uterine horns. Diam. 2.3—3.7 mm	Follicles large	Towards end of stage
II about 6 hrs	Non-nucleated cornified cells only	Maximum distension of uterine horns, of diam. 5 mm. Escape of fluid and regression to 1.8 mm diam.	Follicles largest and ready to burst	+
III about 21 hrs	Cornified cells only in large quantities forming a cheesy mass	Epithelium undergoing degeneration. Uterine horns narrow, diam. abt. 2 mm	Ovulation	0
IV about 6 hrs	Cornified layer. Many leucocytes	Epithelium undergoing degeneration and regeneration. Diam. 2.2 mm	Young corpora lutea. Follicles small	0
V about 57 hrs	Epithelial cells. Leucocytes. Mucus.	Epithelium undergoing regeneration. Diam. 1.7 mm	Follicles growing. Corpora lutea continue to grow	

According to various scientists the blood values are as follows:

Haemoglobin = 85—105 per cent

Red cells = 6.5—10 millions

White » = 7,000—12,000

The index $\frac{\text{Hb} \cdot 5}{\text{Red cells} \cdot 100}$ is low in the rat.

The diameter of the red cells = 6.5—7 μ .

Test Animals used by the Author.

The rats used for this work, in all about 200 animals, were mostly such as had been bred by the hormone laboratories at Lund and Malmö and were of the breed *mus norvegicus*. About fifty per cent of the rats were pure white, so-called "albinos", the others brown-spotted blacks. The two types have proved to be similar from the point of view of sexual biology.

Food. The animals have been fed with mixed food, usually garbage consisting of sausage, meat, cheese, bread, rinds of pork,

fish, etc. Cow's milk distributed twice a week and water the whole time ad libitum. Generally speaking this diet corresponds to the so-called normal rations given at hospitals and should be sufficient with regard to albumen, carbohydrates, fats, vitamins and minerals. At any rate the rats have appeared to thrive, have portrayed a normal sexual cycle and have increased as desired.

Chapter III.

Experimental Anaemia.

In chronic haemorrhagic anaemia not only the body's supply of haemoglobin but also the Hb-content per volume unit of blood is reduced. From that point of view it would seem justified to express the degree of chronic haemorrhagic anaemia in the usual way, stating the haemoglobin value and the number of red cells respectively per unit of volume without measuring the total blood volume.

Anaemia in this paper refers to the condition prevailing when the supply of haemoglobin and red cells is reduced in the organism, insofar as this finds expression in lower haemoglobin values and a reduced number of red blood-corpuscles respectively per unit of volume of blood.

Anaemia can be brought about experimentally in many ways. In experiments with rat's food poor in Fe has often been resorted to above all only a milk diet. HART, STEENBOCK, WADDEL and ELVEHJEM (1928), SCHMIDT (1930), BEARD and MYERS (1931), BEARD and BOGGES (1937), HAMRE and MILLER (1938) and others were able to make rats highly anaemic by using a milk diet only. Their investigations have been concerned with the formation of blood, the part then played by iron and copper respectively, etc.

The anaemia caused by the extirpation of the spleen in rats has been carefully studied.

HEDENIUS (1940) produced severe anaemia in rats and rabbits by repeated intravenous injections of heparin, which is explained by the heparin blocking the supply of prothrombin, thus neutralizing the activating influence of that substance on the hematopoiesis.

Beyond doubt the purest form of experimental anaemia obtainable is haemorrhagic anaemia. Every other procedure to anaemize the organism, whether by the injection of various substances, by the extirpation of the spleen, or by the administration of food

poor in Fe, involves factors that disturb the purity of the experiment.

Food poor in Fe implies an attack on the formation of blood with anaemia as *one* result. For a study of the influence of anaemia on the ovarian function, however, it is necessary to bring about anaemia and nothing but anaemia. Bleeding must thus be resorted to as the most effective method of causing anaemia, if anaemia is to be identical with a reduced supply of haemoglobin and red cells. This, of course, also implies loss of blood serum and the influence of this upon the problem discussed must naturally be investigated.

A few methods for the phlebotomy of rats have been used. If, as in the present work, we desire to keep up the anaemia for several weeks, cardiac punctures and the denudation of large abdominal vessels must be rejected as unsuitable. — It has not been possible to obtain a better blood letting with heparin or dicumarin.

Chapter IV.

Methods.

The experiments were started at a time when the female rats were considered full-grown and had been sexually mature for a few weeks, *i. e.* at an age of about three months. Each test animal was kept in a glass jar. Every morning, between 7 and 8 a. m., vaginal smears were taken. In this way the vaginal cycle of the animals could be checked in detail. Animals showing no regular vaginal cycle for 2—3 weeks were discarded.

Method of examining the blood. A blood count was made once a week on each test animal and sometimes every other day. $\frac{1}{10}$ n HCl was used for the dilution when determining the Hb content, the dilution being 1 : 100. The reading was performed according to Autenrieth-Königsberger's method, three readings being made 20 minutes after the test and the mean then calculated. The source of light was uniform throughout in a dark room according to the procedure outlined by ENGHOF (1937). The same apparatus has been used since 1942 and prior to the experiments as well as during them, it was standardized according to Haldane's standard.

Hayem's solution was used as a diluting medium for the red cell count. 40 rectangles in Burker's counting chamber, so-called "C-rectangles" (Boström 1941), spread over the whole field were counted. As is customary, the blood cells touching two of the sides of the rectangle have been counted.

Control of method. By making double counts in 14 cases an attempt has been made to obtain an estimation of the haematological technique, the same pipettes and counting chambers of course being used. The examiner collected two samples immediately after each other, the samples were mixed by another person, and the determination of the Hb content and the blood count were made by the examiner in the usual manner without his knowing which samples had come from the same rat. The result is seen in Table 2 (p. 12).

Table 2.

Control of the haematological method.

Case	Test 1		Test 2		Difference	
	Hb %	Red cells mill.	Hb %	Red cells mill.	Hb %	Red cells mill.
1	61.5	5.06	63.0	4.83	1.5	0.23
2	60.5	5.22	62.0	5.11	1.5	0.11
3	37.0	4.44	37.0	4.49	0	0.05
4	36.0	4.44	36.0	4.47	0	0.03
5	68.5	4.98	70.0	4.86	1.5	0.12
6	51.5	4.33	51.5	4.30	0	0.03
7	34.5	4.28	34.5	4.11	0	0.17
8	37.0	4.30	37.0	4.33	0	0.03
9	30.0	4.13	28.5	4.06	1.5	0.07
10	34.5	3.81	33.0	3.90	1.5	0.09
11	30.0	4.16	29.0	4.26	1.0	0.10
12	33.0	5.72	34.0	6.17	1.0	0.45
13	30.0	4.17	30.0	4.11	0	0.06
14	37.0	5.00	37.0	4.85	0	0.15
With a probability of 997:1000 the errors in the blood counts are not greater than					2.1 Hb %	0.36. mill.

The results shown in Table 2 have been used as a basis for the statistical calculation of the reliability of the blood counts made. They may be said to suffer from errors which

$$\begin{aligned} \text{for Hb} &= 2.1 \text{ Hb \%} \\ \text{for the red cells} &= 0.36 \text{ mill.} \end{aligned}$$

Method of anaemization. The rat is placed in a "cigar-box" made for the purpose with a slit for the tail. The tail is heated in a bowl of hot water (up to about 50° C). In the hot water stands a graduated glass almost filled with hot water. After the tail has been heated it is carefully dried and a slice about 2 mm thick is cut off with a nipper. The blood required is collected and the tail is placed in the graduated glass. After 10—30 minutes, during which time the temperature of the water must be checked, 2—3—4 ml of blood have been drained into the hot water in the cylinder. The volume is checked before and after the bleeding. This method has been used for the bleeding of all the animals and the anaemia has been satisfactory.

Table 3.

Blood values in 20 animals before and after venesection.

Animal No.	Before bleeding			After bleeding		
	Hb %	Red cells mill.	Index	Hb %	Red cells mill.	Index
15	113	7.3	0.77	34	4.5	0.38
16	111	7.7	0.72	33	4.4	0.38
17	106	7.4	0.72	33	4.7	0.35
18	100	6.8	0.74	34	4.2	0.40
21	96	6.5	0.74	34	3.6	0.47
22	107	7.3	0.73	38	3.7	0.51
23	107	7.5	0.71	31	3.6	0.43
87	107	7.5	0.71	23	3.6	0.35
88	98	6.6	0.74	27	2.9	0.17
89	93	7.0	0.66	25	3.7	0.34
92	94	7.3	0.64	21	2.7	0.39
93	97	7.9	0.61	24	3.6	0.33
100	97	7.8	0.62	16	3.1	0.26
101	100	8.2	0.61	19	3.3	0.29
102	102	7.6	0.67	21	3.5	0.34
103	99	8.4	0.59	22	3.0	0.37
111	99	7.3	0.68	23	2.8	0.41
112	106	8.6	0.62	29	3.9	0.37
113	100	6.8	0.74	32	4.1	0.39
144	94	7.6	0.62	30	4.2	0.36
Means	101.3 ± 1.3	7.46 ± 0.12	0.69 ± 0.112	27.6 ± 1.3	3.66 ± 1.3	0.38 ± 0.014

The degree of the anaemia has naturally been dependent upon the success of the bleeding. In most cases the Hb content has been reduced to 30—40 per cent or less in a few days, and the red cells to 3—4 millions. The hypochromic character of the anaemia was most pronounced towards the end of the blood-letting period, the Hb content then being low the whole time, but the number of red cells increased. This is illustrated in Table 3.

Table 3 shows that the index, which is low even in normal rats, falls considerably during the course of the anaemization, on an average from 0.69—0.38. The decrease is significant.

The formation of blood was intense, which is evident partly from the fact that frequent bleedings were necessary in order to keep the anaemia at a low level and partly from the fact that the reticulosis increased, as shown in Table 4.

Table 4.

*Reticulocyte values in 9 animals.
Increase during anaemia.*

Animal No.	Reticulocytes in ‰		
	At beginning of the test	At end of the test	Increase
A 7	3.1	23.0	19.9
A 13	3.0	34.0	31.0
A 14	3.4	31.0	27.6
A 15	3.2	28.1	24.9
A 16	4.1	26.7	22.6
A 17	3.0	29.0	26.0
A 18	4.0	27.9	23.9
A 20	3.2	27.0	23.8
A 21	9.0	38.0	29.0
			Mean 25.4

In order to keep the anaemia at a fairly constant level it was necessary to bleed each animal every other day. If the interval between each blood-letting is longer, remissions arise, the blood values increase between each blood-letting and it is impossible to speak of an anaemic state in the animals.

The method of blood-letting described is tedious and time-wasting, but I have not been able to find a better method for a lenient blood-letting of the animals. The waste of time is partly compensated by the fact that it is possible to bleed 3—4 rats simultaneously.

A total of more than 5,000 venesections have been carried out on about 150 animals.

Chapter V.

Influence of the Anaemia on the Vaginal Cycle.

With the method described above the following investigations have been made:

69 normal female rats. Daily vaginal smears. Blood-letting every day or every other day. Determination of Hb content and red cell content every week. The results are exemplified in Diagram 1. This trial series will be called the "Normal Group".

Explanation of symbols in the diagrams.

The columns at the bottom of the diagram represent the bleedings, their height showing the quantity of blood collected in ml. The unbroken and the dotted curves record the haemoglobin value and the number of red cells respectively. The middle curve represents the phases of the vaginal cycle. Every hollow corresponds to the met and neg respectively and represents the negative phase of the cycle and every peak represents the oestrus and signifies the positive phase of the cycle. Pro has been placed between neg and pos. There is occasionally a continuous pro—pos phase of varying length; it is called "*Dauer*" in the text. At times the vaginal cycle reaches no further than to pro, as shown in the diagrams. The top curve represents the body weight. Each square represents a change in weight of 5 g.

According to Diagram 1 the animal in question has lost in all about 190 ml of blood after 56 blood-lettings during 3 months. Hb remains at a level of about 40 per cent and the red cells are between 4 and 5 millions. After the blood-letting the values increase. The vaginal cycle disappears after slightly more than one month's anaemization, is absent for 68 days and returns 11 days after the end of the anaemization. During this long dioestrous period, which will henceforward be called "*the anaemically negative phase*", a movement in the vaginal mucous membrane is observed on two occasions, but the stage barely approaches the pro-stage. During the blood-letting period the animal first increases in

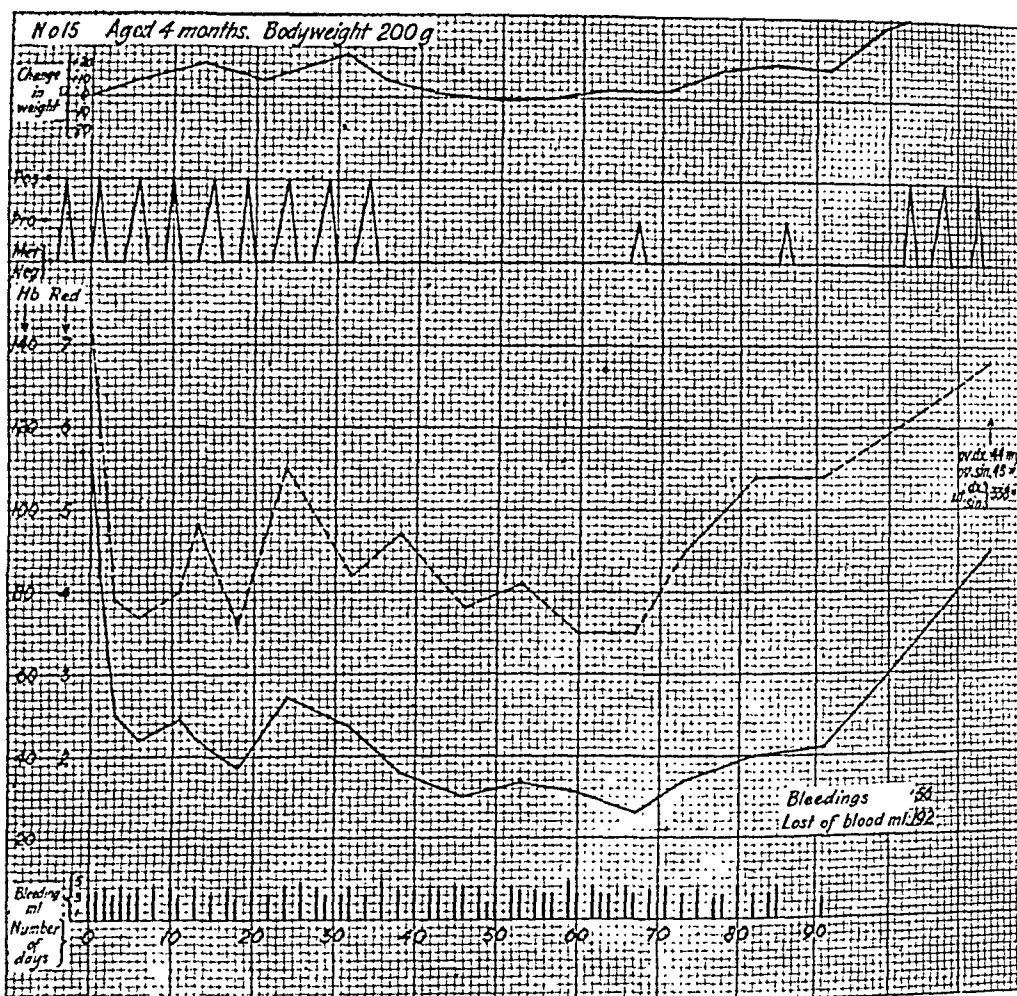


Diagram 1.

weight, then goes down to its original weight, only to increase again toward the end. A great increase in the weight sets in after the cessation of the blood-letting.

The result of the study of the influence of anaemia on the vaginal cycle is shown in Tables 5—8.

In 68 of the 69 test animals the vaginal cycle disappeared during the anaemization for longer or shorter periods.

Suspension of the vaginal cycle. The time that elapsed from the first blood-letting to the suspension of the vaginal cycle varies considerably, but in somewhat more than 70 per cent of the cases, it is from 2 to 5 weeks, which is seen in Table 5 (p. 17). The shortest interval is 4 days, the longest 51. The mean is 27.4

Table 5.

Number of days from the first anaemization to the suspension of the vaginal cycle. Quantity of blood drained during that time.

Number of days	Number of animals	Quantity drained ml	Number of animals
4	1	11-20	3
8-14	6	21-30	15
15-21	15	31-40	13
22-28	14	41-50	13
29-35	19	51-60	9
36-42	3	61-70	3
43-49	8	71-80	7
51	2	81-90	4
		95	1
Total 68		Total 68	
M = 27.4 ± 1.06 days		M = 45.8 ± 2.44 ml	

± 1.06 days. During this time the quantity of blood drained varies considerably from cases to case. This can also be seen in Table 5. In nearly 75 per cent of the cases the blood drained is between 20 and 60 ml. The smallest quantity of blood is 11 and the largest 95, the mean being 45.8 ± 2.44 ml.

Table 6.

Blood values on suspension of vaginal cycle.

Hb %	Number of animals	Red cells mill.	Number of animals
20-24	3	2.0-2.4	1
25-29	9	2.5-2.9	3
30-34	10	3.0-3.4	19
35-39	20	3.5-3.9	20
40-44	12	4.0-4.4	21
45-49	8	4.5-5.0	4
50-54	4		
55-59	1		
60	1		
Total 68		Total 68	
M = 38.0 ± 0.98 %		M = 3.71 ± 0.07 mill.	

Table 7.

Change in weight from beginning of blood-letting to the suspension of the vaginal cycle in 68 animals.

Change in weight, g	Number of animals
+ 15	1
+ 10— + 1	12
± 0	7
— 1— — 10	27
— 11— — 20	18
— 25— — 26	3
Total 68	
$M = -6.5 \pm 1.04$ g	

Degree of anaemia when the vaginal cycle was suspended. In order to answer this question and many others, all the experiments have been recorded on graph-paper, as demonstrated in the diagrams. The weekly examinations of the quantity of haemoglobin and the number of red cells result in curve shown in the diagram, where the probable blood value can be ascertained for the day the cycle is suspended. These blood values for the 68 cases in the normal group have been collected in Table 6 (p. 17).

Table 6 shows that the blood values at the time of the disappearance of the cycle may vary quite considerably. Thus the greatest haemoglobin value is 60 per cent and the lowest 20 per cent, but in more than 85 per cent of the cases the haemoglobin value at the time of the suspension of the cycle was found to be between 25 and 49 per cent. The mean is 38.0 ± 0.98 per cent.

Table 6 also shows the corresponding conditions as regards the red cells. When the sexual cycle is suspended the number of red cells is between 3.0 and 4.4 millions in nearly 90 per cent of the cases, the mean being 3.71 ± 0.07 millions.

Change in body weight on suspension of the vaginal cycle. Table 7 (p. 18) shows that 20 of the 68 rats remained the same weight or experienced but a slight increase. 27 animals experienced a loss of weight of up to 10 g, and 21 rats lost more than 10 g. The mean loss of weight for the whole group is 6.5 ± 1.04 g. The loss of weight is thus not very great. Only 3 of the animals show a loss of weight corresponding to $\frac{1}{8}$ of their original weight.

Table 8.

Duration of the anaemically negative phase in 7 normally anaemic animals. Time from end of the blood-letting to the return of the vaginal cycle.

Animal No.	Duration of negative phase		From end of blood-letting to return of cycle, days	Maximum change in weight during bleeding, g
	Days ¹	Total		
15	68	68	11	+ 26
17	13 + 13 + 19 + 7	52	19	+ 14 - 7
18	9 + 12 + 18 + 44	83	29	- 4 + 22
20	19 + 9 + 12	40	2	- 26
34	20	20	8	+ 6 - 25
A 14	29	29	0 ²	+ 25
A 20	8 + 17	25	10	- 20
Mean 45 days				

¹ As the anaemically negative phase is often interrupted by one or more oestras phases, it is divided into negative periods of varying lengths. The figures in this column show the duration of these periods.

² Cycle returned 7 days before end of anaemization.

The change in weight and its possible influence as an aetiological factor with regard to the changes observed in the ovarian functions will be discussed more thoroughly in Chapter VII.

The anaemically negative phase. After a study of 74 cases LONG and EVANS found that the longest continuous dioestrous phase was 78 hours. Personally I have never found a negative picture longer than 5 days in the control animals for the various series. The same applies to all the 68 animals in the normal group, which present a regular vaginal cycle before the blood-letting and often long after it. In this paper a *continuous met-neg-stage* of at least 6 days is therefore looked upon as an *anaemically negative phase*.

Occasionally the anaemically negative phase was interrupted after 10—14—17 days or more by some positive phase or other, only to be resumed and last as long as it was possible to effect bleeding, *i. e.* as long as there was anything left of the tail.

In 7 cases the anaemically negative phase was allowed to *continue* without any other operations being performed than the blood-letting. These 7 cases, henceforward called "the Normally Anaemic

Group", will be seen in Table 8 (p. 19), which shows, *inter alia*, that the length of the negative phase varies very considerably. No regular variations are reflected in the table. Some time after the cessation of the blood letting, when the blood values increased to normal or above normal in a few days, the cycle returned, which is also seen in Table 8. The animal was ready for breeding.

Pseudo-pregnancy and the anaemically negative phase. For the 7 animals in Table 8 there occur in all 16 suspensions of the vaginal cycle; only 4 out of these 16 continuous dioestrous phases have a length corresponding to that of pseudo-pregnancy.

In 5 cases the female rat was brought together with a male rat during the anaemically negative phase upon heat being produced by artificial means; it was possible to establish the presence of sperms in the vagina, but neither pregnancy nor pseudo-pregnancy occurred. This test, which was also made with another object in view, is described in greater detail in Chapter VII. It demonstrates, however, that the sexual function is influenced in such a way during anaemia, that the artificially stimulated heat in the female and the subsequent coitus with its excitation of the hypophysis-interbrain system, cannot produce pseudo-pregnancy, which so easily arises after sterile coitus.

Control animals. 13 test animals, most of them sisters of the respective test animals in the various series, all showed a normal vaginal cycle.

In none of these control animals did pseudo-pregnancy occur. Thus the dioestrous periods in the test animals cannot possibly be ascribed to pseudo-pregnancy, which is also apparent partly from the histological picture of the vaginal smear, partly from the fact that in most cases the duration of the negative phase does not correspond with the length of pseudo-pregnancy in the rat, which was pointed out above when discussing Table 8, p. 19.

The result of the investigations is that *haemorrhagic anaemia of a certain extent influences the sexual functions of the rat, so that the vaginal cycle is suspended for a varying length of time.*

Chapter VI.

What Factor in the Anaemization Leads to the Change in the Vaginal Cycle?

Haemorrhagic anaemia implies a loss not only of blood cells, but also of blood serum.

The loss of blood serum. In order to find out whether it is the loss of blood serum that is the active factor in the influence of anaemization on the vaginal cycle, the following investigation was made:

8 rats were anaemized. The blood was collected in a centrifuge tube containing 2 ml physiological saline. This tube was used instead of the graduated cylinder and was fixed in the water by means of clamps. Immediately after the anaemization the contents of the tube were centrifugated. The serum and the saline were injected subcutaneously into the test animal, which was thus deprived of practically only the blood cells. The most adequate method would no doubt have been to make an intravenous reinjection of the serum, but this is hardly feasible on a rat the number of times that would be necessary for this experiment. Moreover, the substances dissolved in the serum will, when injected subcutaneously, easily enter the vascular system, with the exception of the serum albumin.

The animals in this test series will henceforward be called "Serum Rats".

The investigation is exemplified in Diagram 2.

After well over a month's anaemization the ovarian cycle disappears, and a continuous dioestrous picture of more than 3 weeks ensues. The intense anaemia does not seriously affect the body weight.

The result of the investigation is shown in Table 9 (p. 23).

Table 9 shows that the difference between the serum rats and the rats of the normal group is very small, and, considering the small number of cases within the former group, it is not significant.

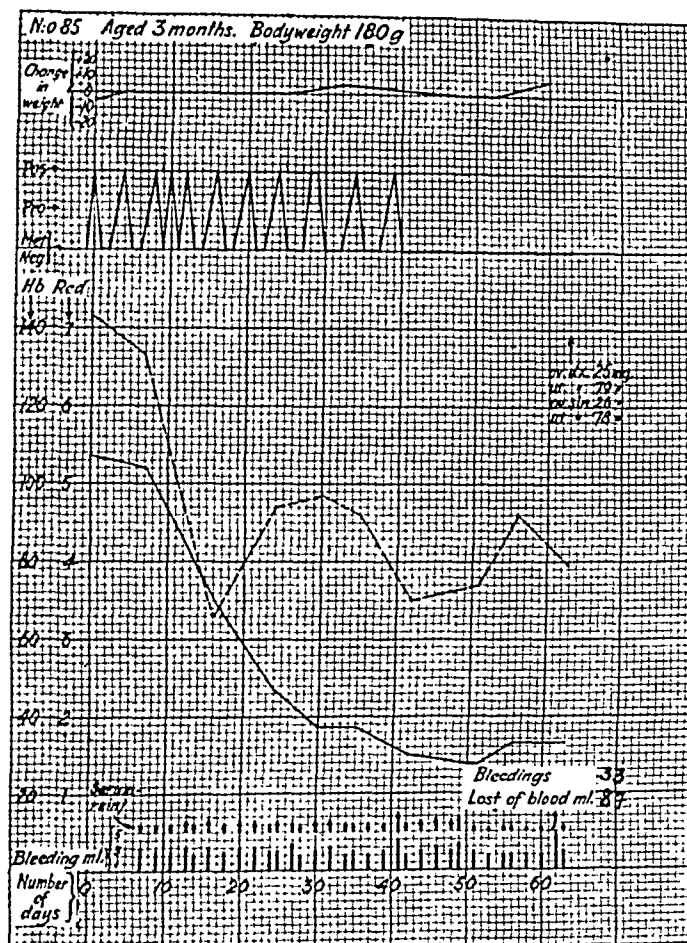


Diagram 2.

In order to establish whether there occurs a reduction in the serum albumin during haemorrhagic anaemia, the serum protein values have been checked in 12 cases according to Kingsley's method. The result of this investigation is seen in Table 10 (p. 23).

It has not been possible to establish any change in the content of serum albumin, at any rate no reduction. The mean for the 12 cases is ± 0.25 per cent, thus a small, not significant increase.

The above investigation would seem to indicate that the loss of serum or any substance therein cannot be made responsible for the influence of the anaemization of the vaginal cycle.

The loss of iron. Haemorrhagic anaemia as well as other forms of so-called secondary anaemia implies a reduction of the haemo-

Table 9.

Status at suspension of vaginal cycle in the serum rats. A comparison between these animals and the 68 in the normal group (Chapter V).

Animal No.	Status at suspension of cycle				
	After number of days	Blood drained ml	Hb %	Red cells mill.	Change in weight g
79	24	39	25	3.7	- 10
80	52	75	29	3.8	- 4
81	12	19	42	3.5	+ 14
82	15	26	54	3.9	+ 25
83	54	87	30	3.9	0
84	37	65	29	3.6	+ 10
85	33	50	32	3.8	+ 4
86	29	45	31	4.4	+ 12
Mean	32.0 ± 3.09	50.8 ± 7.11	34.4 ± 2.86	3.83 ± 0.20	+ 6.4 ± 3.03
Mean for 68 cases belonging to the normal group	27.4	45.8	38.0	3.71	- 6.5

Table 10.

Serum albumin values during anaemia.

Animal No.	Serum albumin in per cent		
	Before test	At end of test	Change
87	6.60	6.80	+ 0.20
88	6.95	7.10	+ 0.15
89	6.45	7.00	+ 0.55
93	6.85	6.40	- 0.45
102	6.30	6.00	- 0.30
75	7.80	7.60	+ 0.60
76	5.20	6.96	+ 1.75
78	6.50	6.80	+ 0.30
94	7.35	5.65	- 1.70
79	6.10	7.25	+ 1.15
80	7.30	7.10	- 0.20
82	6.70	7.70	+ 1.00
			Mean + 0.25

globin content and of the red cells in the organism. It is thus *a priori* very probable that the important factor in the influence of haemorrhagic anaemia in the sexual function is to be sought in the loss of haemoglobin and red cells respectively. It is consequently reasonable to assume that the loss of iron is the principal factor in haemorrhagic anaemia.

In order to study this question substitution tests were made with iron. *Ferri ammonii citras* was chosen as a suitable iron substance.

One ml blood contains about 0.5 mg iron. A test animal deprived of about 3—4 ml blood 4 times a week thus loses maximum 8 mg iron per week.

8 rats were anaemized in the manner previously described. Subcutaneous injections of a 5 % solution of *ferri ammonii citras* were given 4 times a week. Each time 2 mg Fe were administered, i. e. the animal received 8 mg Fe per week. This substitution treatment continued during the whole period of anaemization. It was soon found that the test animals in this series, henceforward called "Iron Rats", bled much more profusely than the others; but that nevertheless it was very difficult to get lower blood values. The test is exemplified in Diagram 3 (p. 25).

Although, as is seen from the diagram, the test animal lost about one-third of all its blood 4 times a week, the haemoglobin did not drop to the level where the vaginal cycle generally ceases. In spite of anaemization for merely 4 months and a total loss of about 290 ml blood, the Hb value could not be reduced by more than about 50 per cent, and only on one occasion did the vaginal cycle show continuous dioestrous picture of 5 days. The loss of weight is maximum 20 g.

The result of the substitution tests with iron is shown in Table 11 (p. 26). For comparison the same data for the 7 normal anaemic cases have been collected in Table 12 (p. 27).

Tables 11 and 12 show that the iron rats suffered a much greater loss of blood, but in spite of this haemoglobin value did not drop at all so far as in the normal cases. During the anaemic period the haemoglobin value of the iron rats is between 60 and 70 per cent as compared with about 40 per cent for the normal group. There is no difference to speak of between the number of red cells in the two groups. The hematocrite value is slightly reduced for the iron rats. There must thus have occurred an enormous formation of blood in the test animals of this group.

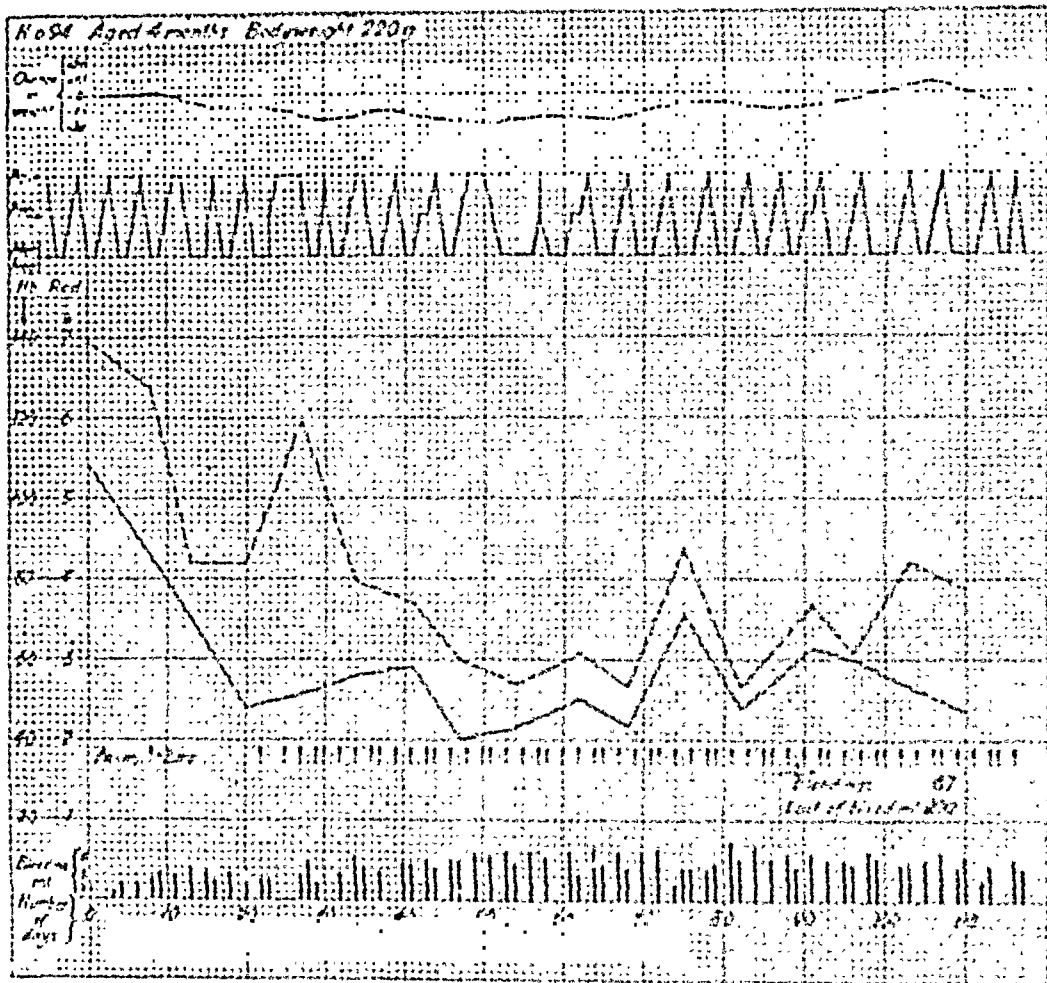


Diagram 3.

The time of observation for the 8 test animals was at least 3 months.

Out of the 8 iron rats, 7 show no very long suspensions of the vaginal cycle. Seven days after the beginning of the anaemization and after losing only 11 ml of blood, one test animal had a negative phase lasting 12 days. The Hb value was then 55 per cent. The suspension of the vaginal cycle was not repeated.

By administering iron it was thus possible in 7 cases out of 8 to prevent the suspension of the vaginal cycle that otherwise always occurs. On the other hand, it was not possible to anaemize these animals to the same degree as the others, although they bled much more than usual.

Table 11.
Blood values and quantity of blood drained in 8 iron rats.

Animal No.	Number of bleedings	Quantity of blood drained ml	Blood values during anaemia						Haematocrite			
			Number of counts	Haemoglobin %			Red cells mill.			Before test	At end of test	Difference
				Max.	Min.	Mean	Max.	Min.	Mean			
70	52	170	10	85	54	70	5.4	3.3	4.3	—	—	—
72	52	164	11	90	48	65	5.3	3.1	4.1	—	—	—
74	64	198	14	90	50	69	6.0	3.1	4.4	—	—	—
75	62	199	13	80	56	70	5.1	3.4	4.5	45	46	+ 1
76	60	193	13	89	53	73	5.4	3.9	4.7	47	50	+ 3
78	61	181	13	77	47	68	5.3	3.5	4.6	47	38	— 9
94	67	290	13	71	40	53	5.9	2.6	5.6	48	40	— 8
98	55	176	10	67	42	58	4.3	2.5	3.7	40	37	— 3
Mean		196				66			4.2			

Table 12.

Blood values and quantity of blood drained in the normal anaemic group (Chapter V).

Animal No.	Number of bleedings	Quantity of blood drained ml	Blood values during anaemia						
			Number of counts	Haemoglobin per cent			Red cells mill.		
				Max.	Min.	Mean	Max.	Min.	Mean
15	56	192	13	54	26	38	5.5	3.5	4.4
17	44	142	7	53	31	42	5.2	3.3	4.3
18	57	197	12	46	27	38	4.8	3.3	3.9
20	42	130	7	57	29	41	4.1	3.5	3.7
34	40	111	7	49	23	39	5.3	2.0	3.8
A 14	28	65	18	62	42	49	4.7	2.6	3.6
A 20	35	75	22	68	40	49	5.2	2.4	3.7
Mean		130				42			3.9
Corresponding means for iron rats		196				66			4.2

As, owing to the great losses of blood, the iron rats lost more serum than any other test animals but nevertheless retained the vaginal cycle, these tests strongly support the conclusion that a loss of serum does not influence the ovarian function.

The result of the investigation can be interpreted in but one way: *It is the loss of the red cells and the haemoglobin resp. or of the iron that causes the influence of haemorrhagic anaemia on the ovarian function.*

One question left unanswered in this paper is whether it is the part played by iron as a catalyzer in the metabolism of the cells or its part in the formation of haemoglobin and the transportation of oxygen that is of most importance.

Chapter VII.

What is the Point of Attach of Anaemia?

The influence now demonstrated to be exercised by anaemia on the ovarian function may be effected in different ways. The anaemia might be supposed to influence the whole organism in such a way that the general state of health deteriorates, consequently impairing the ovarian function.

General state of health during anaemia. It would be reasonable to expect that the degree of anaemia with which we are concerned here would considerably reduce the strength of the animal. But this does not seem to be the case. Some of the test animals do look rather taken immediately after a large venesection, but they pick up very quickly; after only a few minutes they run around as usual, have a good appetite, etc. Their fur does not change during the anaemization.

The body weight of laboratory animals is a sensitive gauge of their condition.

Table 18.

Change in weight in the normally anaemic group.

Animal No.	Body weight at beginning of test, g	Change in weight	
		At suspension of cycle, g	At conclusion of anaemization, g
15	210	+ 15	+ 20
17	215	0	- 7
18	230	+ 6	+ 13
20	182	- 16	- 4
34	204	0	- 25
A 14	240	+ 5	+ 24
A 20	205	- 15	- 20
		Change in weight for the whole group	+ 57 g - 56 ?
		Total + 1 g	

Table 14.

Total change in weight for 68 anaemized animals (normal group, Chapter V).

Change in weight, g	Number of animals
+ 25—+ 16	7
+ 15—+ 6	10
+ 5— 4	12
— 5— 14	15
— 15— 24	18
— 25— 34	3
— 35— 44	9
Total 68	
$M = -5.2 \pm 2.03 \text{ g}$	

Table 15.

Change in weight and vaginal cycle of 18 control animals.

Animal No.	Body weight		Increase in weight g	Vaginal cycle
	At beginning of experiment	At end of experiment		
6	165	180	15	Normal
7	180	190	10	Normal
14	210	245	35	Dauer 5 + 9 days
19	196	212	16	Normal
24	222	248	26	Normal
29	230	245	15	Normal
137	169	190	21	Normal
138	191	208	17	Normal
139	223	225	2	Normal
140	176	190	14	Normal
147	180	205	25	Normal
148	183	206	25	Normal
A 5	—	—	23	Dauer long
Mean increase = 18.8 ± 2.29				

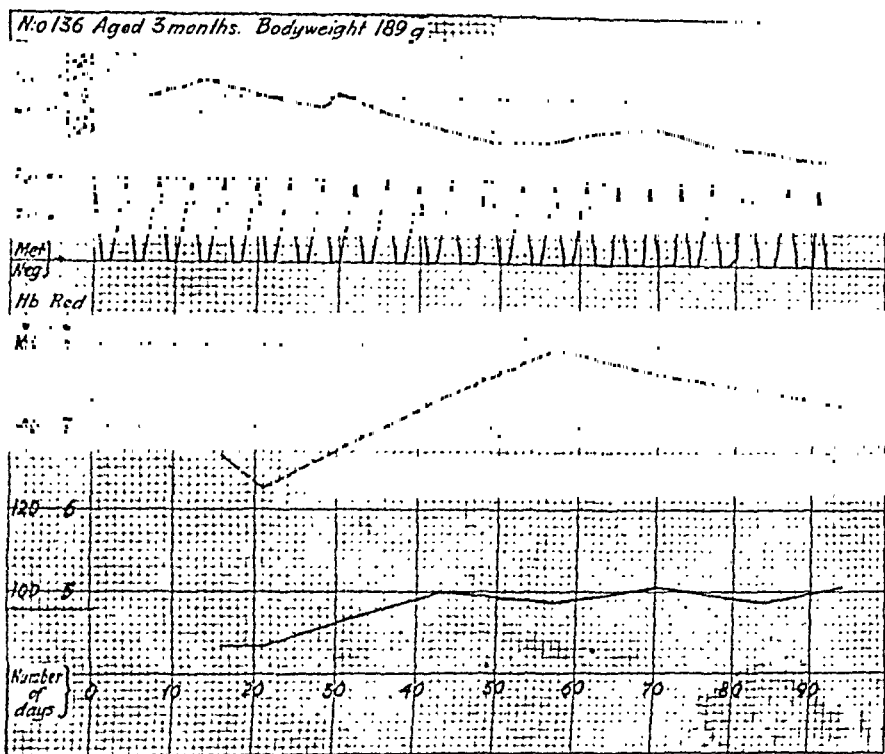


Diagram 4.

The body weight during anaemia. Table 7, p. 18, shows that the loss in weight at the time of the beginning of the anaemically negative phase is on an average of 6.5 g for each of the 68 anaemized animals in the normal group.

Table 13 (p. 28) shows the *total* change in weight during the whole anaemization period for the 7 test animals not subjected to any other operations than blood-letting (normally anaemic group). The table shows that for the whole group the difference between the weight at the beginning of the experiment and the weight at its end is very small.

As is shown in Table 14 (p. 29), the loss of weight for all the 68 animals of the normal group at the end of the anaemization averaged 5.2 g.

The change in weight in the 13 control animals is shown in Table 15 (p. 29).

While the test animals go down about 6 g in weight, the control animals go up on an average 19 g.

Influence of starvation. It has been shown above that the test animals' loss in weight is not very large, being only a few

Table 16.

Change in weight and vaginal cycle of 7 "starved rats" and 4 control animals.

Time of observation 3 months.

Animal No.	Body weight		Change in weight g	Vaginal cycle	Remarks
	At beginning of experiment g	At end of experiment g			
129	212	172	- 40	Normal	Dauer 16 days neg. 6 days neg. 12 + days
131	226	185	- 41	Normal	
132	249	210	- 39	Normal	
133	212	187	- 25	(Normal)	
134	205	175	- 30		
135	211	175	- 36	Normal	
136	189	150	- 39	Normal	
Control animals	137	169	+ 21	Normal	
	138	191	+ 17	Normal	
	139	223	+ 2	Normal	
	140	176	+ 14	Normal	

per cent of the original weight of the animal at the beginning of the experiment, but most of the test animals nevertheless show a small decrease in weight. Can this be in some way connected with the changes observed in the vaginal cycle?

7 rats were starved, 4 of their sisters served as control animals and were given the ordinary quantity of food. The food for the 7 test animals was of the usual quality, but the quantity was reduced so that the weight of the rats was slowly *pressed down* at least as much as would be the result of *anaemization*. The investigation is exemplified in Diagram 4.

The test animal in Diagram 4 shows somewhat increased blood values during the period of observation. The vaginal cycle is quite normal, although the weight was successively reduced no less than 35 g.

Table 16 presents all experiments in this group together with the control animals.

During a period of 3 months the weight of the test animals was reduced about 40 g, *i. e.* considerably more than in the case of the anaemia animals, while for the 5 control animals the weight

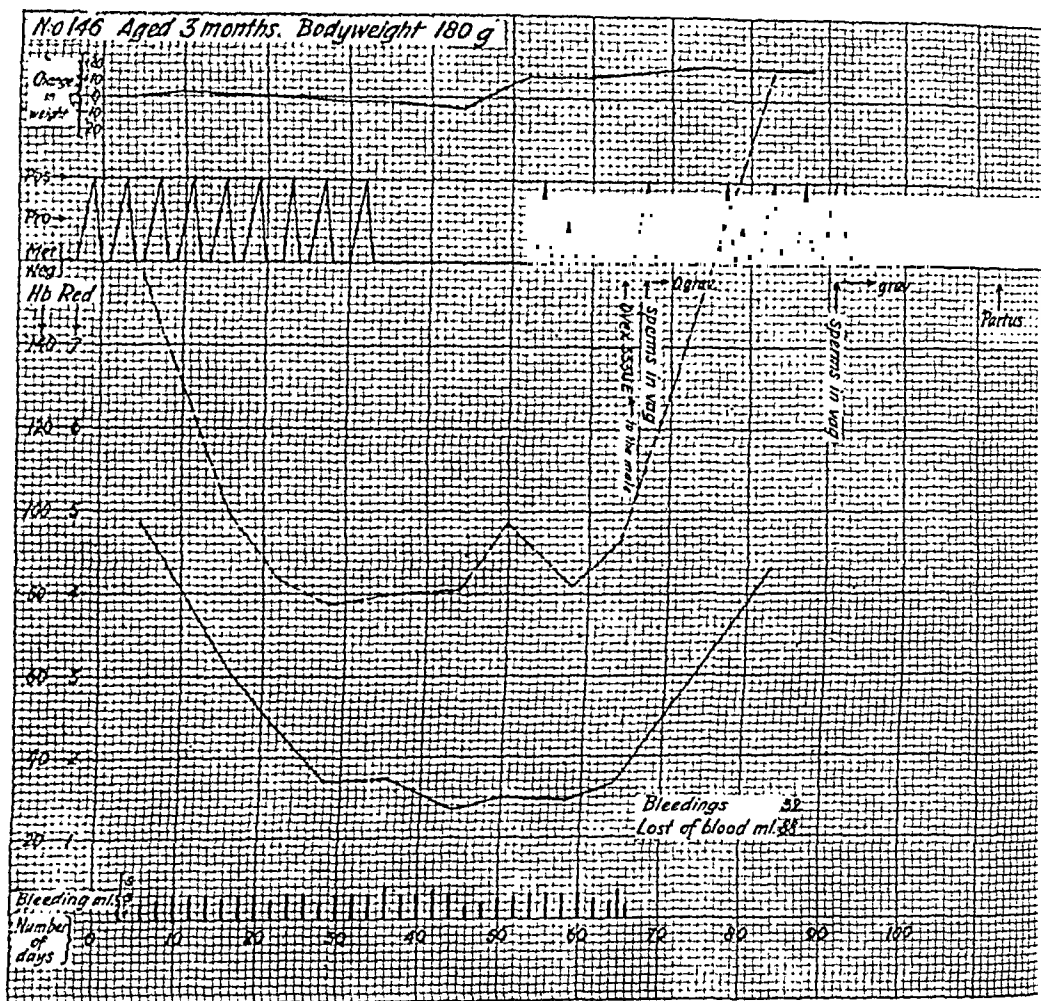


Diagram 5.

increased by almost 20 g during the same length of time. Six weeks and $2\frac{1}{2}$ months respectively after the beginning of the experiment the vaginal smears of one of the 7 rats mirror a long dioestrous phase (it should be noted that this very rat, No. 134, suffered a considerable loss of blood at a blood count). Towards the end of the test another rat shows a negative phase of 6 days. All the other test animals display an absolutely regular vaginal cycle.

The experiment indicates that the great loss of weight is in most cases of no vital importance to the function of the ovary during a period of observation of 90 days.

An experiment intended to further illustrate the importance

of the general condition was carried out in the following manner: 5 rats were anaemized, 2 controls. During the anaemically negative phase obtained, oestrogenic substance was injected in such doses that the vaginal smears showed pro and pos respectively; in other words artificial heat was produced. When the vaginal smear is pro in the morning it is probable that the animal will be in heat on the evening of that day or during the night following. The female is then placed with the male. By examining the vaginal smear for sperms or a vaginal plug it is possible to establish whether or not coitus has taken place. The test is exemplified in Diagram 5 (p. 32).

After 27 days of anaemization the animal in Diagram 5 has a continuous negative phase lasting 3 weeks. In the next anaemically negative phase 333 IU oestrogenic substance (*guttae ovox Leo*) are injected on the 7th day. The answer comes a couple of days later, the female is placed with the male, and sperms are found in the vagina. The anaemization is then stopped. Eleven days later, 9 days after coition, the vaginal cycle returns. In the 4th pro-pos phase the female is again placed with the male and immediately becomes pregnant.

The 5 cases in this trial series will be found in Table 17 (p. 34).

Table 17 shows that in all 5 cases the female rat accepts coitus during the anaemically negative phase if it is interrupted by artificial oestrus. The table also shows that neither pregnancy — which of course was not expected, seeing that the positive vaginal smear was not the result of normal ovulation — nor pseudo-pregnancy has occurred in any instance. The importance that can be ascribed to the fact that no pseudo-pregnancy resulted has been pointed out in Chapter V. At the spontaneous heat arising shortly after the end of the anaemization, the rat becomes pregnant and has a normal delivery.

None of the investigations discussed in this section of Chapter VII indicates that the influence of anaemia on the ovarian function is due to deterioration of the general state of health.

Function of the mucous membrane of the vagina during anaemia. As is well known, sideropenia is characterized by certain symptoms of insufficiency on the part of various mucous membranes. As already pointed out, iron plays a very important part in the metabolism of the cell. The thought can thus not be dismissed that owing to lack of iron the anaemia influences the vaginal epithelium in such a way that it suffers damage and cannot react in a

Table 17.

During artificially effected heat during an anaemically negative phase the female accepts coition, but becomes neither pregnant nor pseudo-pregnant. During spontaneous heat after the cessation of anaemia all the animals become pregnant except one control animal, which becomes pseudo-pregnant.

Animal No.	Anaemically negative phase					After return of cycle						
	Anaemi- cally neg., days	Pro and pos. resp. after how many days	Sperms and plugs, resp., in vagina	Duration of artificial heat in days	Number of days smear remains neg.	Preg- nancy	Pseudo- preg- nancy	Placed with male after how many normal cycles	Sperms and plugs, resp., in vagina	Preg- nancy	Pseudo- preg- nancy	Partus
141	12	1½	sp. + +	2	6	0	0	3	sp. + +	+		+
142	22	1½	plug	1	8	0	0	2	sp. + +	+		+
143	13	2	sp. +	1	8	0	0	1	sp. + +	+		+
144	8	1½	plug	2	3	0	0	4	sp. +	+		+
146	6	2	sp. +	2	9	0	0	3	plug	+		+
Control animals { 147 148	↑ 33½ IU ↑ Placed ↑ Anaemization Ovaries with male ceases Cycle normal all the time.							23	sp. +	+		+
								23	sp. + ?	0	+	

normal manner to the impulses from the ovary, which with its secretion of estrogenic substances rhythmically influences the uterus and the vagina. Considering this, it would be desirable to investigate whether the vaginal epithelium reacts to the injection of estrogenic substances. It is known that the administration of estrogenic substances to a newly castrated female can cause the appearance of cornified cells in the vaginal smear and of swollen and transparent uterine horns with serous liquid. This latter phenomenon indicates that an adequate dose of estrogenic substance has been supplied.

11 animals were anaemized. During the anaemically negative phase subcutaneous injections of estrogenic substance, dose 100 IU *ovex*, are made at various times after the cessation of the cycle.

16 such experiments were made on 11 animals. The results are shown in Table 18 (p. 36).

In Table 18 positive vaginal smears have been obtained in 11 out of 16 cases. Laparotomy was performed on 9 animals, and all had transparent uterine horns. In 4 cases the vaginal mucous membrane showed a distinct reaction, but did not go as far as nuclear cells only or cornified cells only. As is the case in the German literature, this indicated reaction is called "Reiz". In one case only does the vaginal smear remain negative.

For checking purposes 100 IU *ovex* were injected in 13 cases on 7 newly castrated female rats. The *ovex* injections were made 1—4 weeks after the castration. The result is shown in Table 19 (p. 37).

Table 19 shows that in 7 cases out of 13, the vaginal mucous membrane reacts with pos. In the 4 cases out of 7 where laparotomy was performed, the uterine horns were transparent. In 5 cases the vaginal smear displayed distinct reaction (Reiz), but cannot be said to be either pro or pos. In one case, finally, no reaction could be found in the vaginal smear.

There cannot very well be a deterioration in the function of the vaginal mucous membrane of a newly castrated rat. The smallest dose of estrogenic substance that can produce cornified cells in such a test animal may thus be said to correspond to the smallest secretion of the ovaries under normal conditions with the same effect on the vaginal mucous membrane. A comparison between Tables 18 and 19 shows that the anaemically negative animals react to the same dose of estrogenic substance

Table 18.
Reaction of vaginal smear and uterus to injection of estrogenic substance during anaemically negative phase.

Animal No.	Number of days anaemically negative	Guttae Ovar IU	Pos. after No. of days	Transparent uterine horns	Thickness of uterine horns in mm	Remarks
36	11	100	1 $\frac{1}{2}$	+		3 weeks after 1st injection. Does not reach pos.
36	15	100	2 Reiz			
38	11	100	1 $\frac{1}{2}$	+		Does not reach pos. 6 days after 1st injection.
37	16	100	1 $\frac{1}{2}$	+		
34	14	100	1 Reiz			Does not reach pos. 7 days after 1st injection.
34	20	100	$\frac{1}{2}$	+		
47	7	100	1 Reiz			Does not reach pos. 5 days after 1st injection.
39	7	100	$\frac{1}{2}$			
39	6	100	1 $\frac{1}{2}$	+	4	7 days after 1st injection. Does not reach pos.
48	6	100	2 Reiz	+		
48	11	100	2	+	4	5 days after 1st injection.
49	8	100	—			
49	13	100	1	+		5 days after 1st injection.
50	9	100	1 $\frac{1}{2}$	+		
51	8	100	1 $\frac{1}{2}$	+	4.5	
69	10	100	1	+		

Table 19.
Reaction of vaginal smear and uterus to injection of estrogenic substance after castration.

Animal No.	Days after castration	Guttae Ovx IU	Pos. after No. of days	Transparent uterine horns	Thickness of uterine horns in mm	Remarks
46	33	100	2			Does not reach pos. " " " " " " " " " " " " " " " "
29	5	100	2			
26	18	100	—			
36	7	100	2 Reiz			
36	22	100	2 Reiz			
36	27	100	2 Reiz			
38	7	100	1 Reiz			
38	22	100	2 Reiz			
38	27	100	2	+	3	
34	7	100	3	+	3	
34	22	100	2 $\frac{1}{2}$	+	3.5	
37	7	100	1	+		
37	22	100	1	+		

Table 20.
Weight of the ovary.

Healthy rats		Anaemic rats		Serum rats during anaemia	
Weight of ovary, mg	Number of cases	Weight of ovary, mg	Number of cases	Animal No.	Weight of ovary, mg
20—24	3	12—16	7	79	13+15
25—29	5	17—21	19	81	18+22
30—34	24	22—26	17	82	29+31
35—39	10	27—31	11	84	27+27
40—44	10	32—36	4	85	25+26
45—49	4	37—41	3	86	30+30
Total 56		Total 61		Mean 24.4 ± 1.77	
Mean 34.8 ± 0.80 mg		M = 23.9 ± 0.83 mg			
		D = 10.9 ± 1.15 mg			

just as readily as the newly castrated animals; the vaginal mucous membrane in the anaemic animals thus reacts in the same efficient way from a hormonal point of view, as in the castrated animals. From this it may be concluded that during the anaemically negative phase the vaginal epithelium retains its ability to react. Consequently *the anaemia does not cause an injury of the vaginal epithelium resulting in the anaemically negative phase.*

Ovarian changes during anaemia. *A priori* it would seem probable that it is the ovaries themselves that are attacked by the anaemia with the result that their incretion is hampered. One of the measures taken to solve this problem was to study the weights of the ovary and of the uterus.

56 healthy and 61 anaemic animals. Extirpation of one of the ovaries, always effected during negative and anaemically negative phases, respectively. The weight of the ovary is ascertained on a torsion balance. The result is shown in Table 20.

Table 20 shows that in nearly 80 per cent of 56 healthy animals, the weight of the ovary is between 30 and 44 mg.

The same table shows that in 61 cases the weight of the ovary during anaemia is much lower than in the healthy group. The mean for the healthy group is 34.8 ± 0.80 mg, for the anaemic group 23.9 ± 0.83 mg. The difference between the two groups, 10.9 ± 1.15 mg, is significant.

In Chapter VI above, where the experiment with serum rats was discussed, data on the weights of the ovaries are lacking, as it was desired to have these weights in one place. For this reason the weight of the ovaries of the serum rats has also been included in Table 20. The mean is 24.4 ± 1.77 mg, *i. e.* about the same value as obtained for the anaemic group.

During anaemia the ovary thus loses about 10 mg of its weight, i. e. almost one-third of its normal weight.

Compensating hypertrophy of the ovary during anaemia. If one of two bilateral organs is extirpated the remaining organ becomes hypertrophic. This is particularly true of the ovary. If one is removed, the other often even doubles its weight. This compensating hypertrophy is a phenomenon dependent on the ovary being quite healthy. A study of the compensating hypertrophy during anaemia thus affords an opportunity to form an opinion of the reaction of the ovary during anaemia.

17 healthy animals. By making an abdominal incision about 1 cm long (the animal anaesthetized with ether) it is possible to study the genitalia; one of the ovaries is measured with a micrometer, it is extirpated and weighed on the torsion balance. The other ovary, which is allowed to remain, is also measured, in order to obtain an idea of its approximate size, whereby it is possible to make an approximative comparison of the two ovaries. When it has been established that the cycle continues, which it does without any interruption whatever, the animal is anaemized and the cycle gradually disappears. At the end of the period of anaemization the remaining ovary is extirpated, and its excess weight thus gives an idea of the compensating hypertrophy during anaemia. The experiments are collected in Table 21 (p. 40).

Table 21 shows that the average compensating hypertrophy during anaemia is 3.2 ± 2.7 mg. The small difference in weight is not significant.

In 4 control cases, also found in Table 21, the average excess weight of the remaining ovary was 25.0 ± 6.2 mg.

In order to examine still more thoroughly the conditions prevailing during compensating hypertrophy, another series was studied as follows.

14 animals were anaemized. The vaginal cycle disappeared. One of the ovaries was extirpated, having been subjected to anaemia. The anaemizations were discontinued and the other ovary was allowed to develop.

Table 21.

Compensating hypertrophy.
 First ovary before anaemization.
 Second ovary during anaemization.

Animal No.	Right ovary before anaemization, weight mg	No. of days between extirpations of ovaries	Left ovary during anaemization, weight mg	Difference mg
A 37	36	75	48	+ 12
A 16	32	57	42	+ 10
A 22	34	50	34	± 0
A 23	34	53	30	— 4
A 24	32	67	20	— 12
A 25	32	64	18	— 14
A 26	38	63	23	— 15
A 27	33	81	46	+ 13
A 38	20	92	38	+ 18
A 31	28	86	47	+ 19
A 32	40	77	45	+ 5
A 33	38	68	30	— 8
A 34	32	68	42	+ 10
A 35	30	94	42	+ 12
30	49	85	46	— 3
31	42	74	46	+ 4
33	31	56	38	+ 7
			Mean	+ 3.2 ± 2.7
Control animals				
A 2	38	80	50	+ 12
A 5	30	81	64	+ 34
A 39	27	39	64	+ 37
29	33	29	46	+ 17
			Mean	+ 25.0 ± 6.2

The 14 cases in this series are found in Table 22 (p. 41).

As might have been expected the compensating hypertrophy is very great in this group. According to Table 22 the mean is 34.4 ± 3.0 mg.

For the sake of clarity, finally, the results of all the studies of the compensating hypertrophy are shown in Table 23 (p. 41).

Table 22.

Compensating hypertrophy.

First ovary during anaemization.

Second ovary at time of regained normal state.

Animal No.	Right ovary during anaemization weight mg	No. of days between extirpation of ovaries	Left ovary again healthy, weight mg	Difference mg
A 7	20	27	54	+ 34
A 14	31	70	84	+ 53
A 17	24	81	54	+ 30
A 18	26	20	58	+ 32
A 20	33	81	64	+ 31
A 6	24	46	70	+ 46
25	41	33	60	+ 19
26	24	39	57	+ 33
27	31	72	56	+ 25
28	31	43	58	+ 27
A 8	23	76	44	+ 21
A 9	27	46	62	+ 35
A 11	26	42	84	+ 58
A 12	20	20	58	+ 38
Mean.				34.4 + 3.0

Table 23.

Compensating hypertrophy.

a) in normal state	25.0 \pm 6.2 mg
b) in recovered normal state after anaemization	34.4 \pm 3.0 „
c) during anaemization	3.2 \pm 2.7 „

While normally the excess weight of the remaining ovary is 25.0 mg, the compensating hypertrophy fails to appear if the animal is so deeply anaemized that the vaginal cycle disappears.

Weight of uterus during anaemization. Deterioration of the ovarian function, whether dependent upon true atrophy of the organ or not, should be mirrored in the uterus, the reactive organ of the ovary, and in its weight.

16 healthy and 36 anaemic animals. One or both uterine horns were extirpated during negative and anaemically negative phases

Table 24.

Weight of uterine horn.

16 healthy animals.

36 anaemic animals.

Healthy animals		Anaemic animals	
Weight of uterine horn mg	Number of cases	Weight of uterine horn mg	Number of cases
121—140	2	59	1
141—160	0	61—80	8
161—180	4	81—100	7
181—200	1	101—120	7
201—220	1	121—140	8
221—240	0	141—160	2
241—260	5	161—180	2
261—280	1	181—200	0
281—300	2	205	1
Total 16		Total 36	
M = 216 ± 14.4 mg		M = 110 ± 5.7 mg	
D = 106 ± 15.5 mg			

respectively, the horn always being removed at the distinct limit where it joins the cervix. Weighed on a torsion balance. The result of this examination is shown in Table 24.

Table 24 shows that in normal animals the weight of one uterine horn during the negative phase of the cycle averages 216 ± 14.4 mg. The corresponding weight during an anaemic stage is 110 ± 5.7 mg, thus only about half the weight of the uterine horn in a normal state. The difference between the two groups, 106 ± 15.5 mg. is significant.

It is hardly possible to explain the very distinct difference in the weight of the uterus in a normal state as compared with its weight in an anaemized state otherwise than that *the endocrine activity of the ovaries is impaired during anaemia*, all the more as it has proved simple to stimulate the uterus during the anaemically negative phase by the administration of estrogenic substances.

Histological examination of the ovaries. The influence exercised by anaemia on the ovary as demonstrated above should be

mirrored in the histological picture of the ovary. In 61 anaemic and 9 healthy animals the ovary was extirpated during the anaemically negative and negative phase respectively. It was fixed in Carnoy-alcohol or Susa. Dyed with haematoxylin-eosin and azan respectively. In most cases the ovaries were cut in serial sections.

In studying these sections it is not possible to find any qualitative difference between the ovaries from healthy and from anaemic rats. Primordial follicles and maturing follicles of all sizes look the same in the ovarian sections from anaemic animals as in those from normal animals. The theca cells also look the same and corpora lutea in various stages of development are found in both groups. Neither has it been possible to ascertain any difference in the structure of the stroma.

A study of the histology of the ovary thus discloses no qualitative change that might correspond to the anaemically negative phase. On the other hand, the normal appearance of the ovaries in the anaemic cases does not preclude changes in the ovarian function. WESTMAN (1942) has demonstrated that histologically quite normal ovaries in women have had a reduced ovarian function.

One question that must be left open here is whether a quantitative analysis of the number of follicles and corpora lutea in the ovary and of the morphology of the cells can disclose what changes in the ovary dictate the influence of anaemia upon the ovarian function.

The studies reported above of the weight of the ovaries and the uterus during normal and anaemic stages indicate that the ovary is greatly influenced by anaemia.

Is the ovary influenced by anaemia via the hypophysis? Since the endocrine activity of the ovary is impaired during anaemia, the question arises whether the impairment is primary or secondary. The relation of the ovary to the anterior lobe of the hypophysis necessitates an examination as to whether the damage to the ovary during anaemia occurs via the gonadotropic function of the anterior lobe of the hypophysis.

9 normal and 14 anaemized animals. During negative and anaemically negative phases, respectively, the hypophysis is extirpated and weighed on a torsion balance. The result of these investigations is seen in Table 25 (p. 44).

The mean weight of the hypophysis in the 14 anaemic cases is 7.61 mg. The hypophysis of the non-anaemized rats is slightly

Table 25.

Weight of hypophysis in 9 healthy and 14 anaemized animals.

Healthy animals			Anaemized animals		
Animal No.	Body weight g	Weight of hypophysis mg	Animal No.	Body weight g	Weight of hypophysis mg
80	245	9.0	87	200	7.5
83	245	9.0	88	178	8.0
114	157	8.5	89	160	6.0
115	190	9.0	92	164	5.5
116	170	10.0	93	174	6.5
117	173	8.0	96	158	8.0
150	—	8.0	100	135	6.5
151	—	8.0	101	163	8.0
152	—	9.0	102	192	8.0
—	—	—	103	158	8.5
—	—	—	106	199	8.0
—	—	—	111	190	7.0
—	—	—	112	190	9.0
—	—	—	113	260	10.0
Mean	197	8.72	Mean	180	7.61

heavier than that of the anaemic animals. The difference, 1.11 mg, is not significant, however.

It is of course not possible to say anything about the function of the hypophysis on the basis of this study of its weight. The number of cases is small and the method rough. If possible the problem must be attacked by testing the function of the hypophysis.

If the suspension of the vaginal cycle during anaemia is due to the function of the hypophysis being impaired, it would not be possible for the hypophysis to cause the usual cyclic changes in the ovary and the vagina during anaemia. In order to ascertain whether this is the case a study can be made of the gonadotropic effect of implanted pituitary glands.

A hypophysis from a test animal in an anaemically negative phase is implanted on an infantile mouse and it is investigated whether the implanted hypophysis can cause the follicles in the ovaries to mature and cause oestrous changes in the vagina. The same experiment is carried out on control animals.

Table 26.
Gonadotropic effect on infantile mouse of hypophysis from 7 anaemic and 4 healthy animals.

Gonadotropic effect on infantile mouse of hypophysis from 7 anaemic mice										
Animal No.	Test animal		Infantile mouse 100 hrs later							
	No. of days anaemically negative	Weight of hypophysis in mg	Vaginal smear		Weight of uterus in mg		Maturity of follicles			
			Impl. mouse	Control	Impl. mouse	Control	Impl. mouse	Control		
87 101 102 103 111 112 113	11 + 20	7.5	pos.		26			++		
	10 + 18	8.0	pos.		20	12		++	0	
	25 + 13	8.0	pos.	neg.	20	11		+	0	
	31	8.5	pos.	neg.	15			++		
	15 + 14	7.0	Reiz.		31	10		++	(+)	
	13 + 19	9.0	pos.					++		
	17 + 14	9.0	pos.					++		
Control animals 114 115 116 117		8.5	pos.		14	7		++	0	
		9.0	Reiz.	neg.	13	15		0	0	
		10.0	pos.	neg.	30			++		
		8.0	pos.		22			++		

↑ Implantation of hypophysis.

It is well known that implantation has been used with a view to obtaining a biological test of the content of gonadotropin in various tissue substrata. The smallest quantity of gonadotropin able to cause in the infantile mouse the changes described above is called 1 MU.

7 rats were anaemized. When the cycle had been gone for 4—5 weeks, the animal was killed. The hypophysis was extirpated, weighed and implanted in the thigh of an infantile mouse weighing about 8 g. By studying the ovary uterus of the mouse it was then possible to establish whether or not there was any gonadotropic effect. In some cases a sister of the infantile mouse served as a control, no hypophysis being implanted. The result of this investigation is shown in Table 26 (p. 45).

6 of the 7 pituitary glands from anaemized rats gave a positive reaction in the vaginal smear of the infantile mouse, and in one case only was the vaginal reaction incomplete (Reiz).

In all cases examined the weight of the uterine horns increased, in some cases considerably. The microscopical picture of the ovaries, finally, disclosed the follicles to mature in a characteristic way in all 7 cases.

Table 26 also shows that the glands of the 4 control animals caused similar changes. In the control group, too, there is one case where the vaginal reaction could not be stimulated higher than to Reiz. In this case the follicles did not mature.

In the 5 cases where a sister of the infantile mouse served as control, the weight of the uterine horns was considerably lower than of the test animals and corresponded to the weight normal for the age.

As the anaemically negative phase in the vaginal smear had remained for several weeks, it seemed reasonable to expect that castration cells would be found in the hypophysis of such animals. In 7 cases the glands of anaemic rats were examined histologically. No castration cells were found.

It has not been possible to establish any qualitative difference between the histological picture of the hypophysis of healthy and anaemic animals.

This method of studying the hypophysis and its function during anaemia has disclosed *nothing to indicate that the observed disturbance of the endocrine activity of the ovary is conditioned by an impaired gonadotropic function. The influence of anaemia on the ovarian function would thus not seem to be transmitted by the hypophysis.*

Summary.

The author has made a study of the influence of haemorrhagic anaemia on the ovarian function of the rat.

The method of anaemizing rats elaborated by the author is time-wasting but effective and as lenient as possible for the animal. A thin slice of the tail is cut off with a nipper, the tail then being submerged in a graduated glass containing hot water. The rat bleeds profusely and the blood drained can be measured. In that manner it has been possible to cause quite a considerable anaemia in the test animals throughout a period of 2—3 months.

In 68 of the 69 anaemized animals, the ovarian cycle was suspended for longer or shorter periods. An "anemically negative" phase of varying duration set in. During this phase the female rat accepted coitus on artificial heat being produced, but did not become pregnant, not even pseudo-pregnant. The change in the vaginal cycle caused by the anaemia is reversible. The ovarian cycle returns some time after the end of the anaemization. The female then accepts coitus with subsequent normal pregnancy and delivery.

The factor of the anaemia that exercises this influence on the ovarian function does not seem to be the loss of any substance in the blood serum, as it has not been possible to eliminate the effect of the anaemia on the ovarian function by a reinjection of serum in 8 test animals. Moreover, in 9 cases examined it was not possible to establish lower serum albumin values during anaemization.

On the other hand, it is evidently the loss of red blood cells and haemoglobin resp. or of the iron that is of vital importance in the anaemization. In 7 out of 8 anaemized rats it was possible to prevent the changes in the ovarian cycles by subcutaneous injections of *ferri ammonii citras*.

A number of observations would seem to indicate that the changes in the ovarian cycle during anaemia cannot be ascribed to general disturbances, but are of a more specific character.

Thus the body weight is but slightly reduced during anaemia and the general condition of the animals is good. Furthermore the female accepts coitus on artificial heat being produced. Animals that were starved so that the body weight went down quite considerably displayed a regular ovarian cycle.

During the anaemically negative phase the vaginal mucous membrane responds to the administration of estrogenic substances. The suspension of the vaginal cycle during anaemia can thus not be due to damage to the vaginal epithelium by the sideropenia brought about by haemorrhagic anaemia.

During anaemia the ovary loses about one-third of its normal weight. The compensating hypertrophy of the remaining ovary normally occurring as a result of unilateral ovariectomy fails to appear during anaemia. The uterus, the reactive organ of the ovary, loses much weight during the anaemia period, but it is stimulated to full development by the administration of estrogenic substances. All these experiments bear out the theory that anaemia attacks the ovary.

This influence on the ovary may be direct or via the hypophysis. The implantation of hypophysis from anaemically negative animals on an infantile mouse causes the same reaction as the implantation of hypophysis from non-anaemic animals. *viz.* oestrus with all its manifestation in the ovary, the uterus and the mucous membrane of the vagina. This supports the assumption that anaemia attacks the ovary direct and not via the hypophysis.

A study of the histological picture of the ovary discloses that there is no manifest qualitative difference between the ovaries from normal and from anaemized animals.

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THE PHYSIOLOGICAL EFFECTS OF TIME SCHEDULE WORK ON LUMBER-WORKERS

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Preface

The present work is part of an investigation on the physiological effects of lumber work which was begun in 1941 and continued during the years 1943 to 1945. As the work had medical as well as forestry and technical aspects, it required real team work, and it is a great pleasure to me to extend my thanks to all those with whom I had the pleasure of cooperating.

The Chief of the *Swedish Committee of Industrial Physiology (Arbetsfysiologiska Undersökningen)*, Professor Y. ZOTTERMAN, who first introduced me to problems of industrial physiology, has constantly facilitated the experimental work with stimulating advice and active help. For all this I wish to express my heartfelt thanks.

I am also deeply grateful to the Chief of the Physiological Department of *Gymnastiska Centralinstitutet*, Professor E. HÖRWIG CHRISTENSEN, whom I had the favour of consulting on several occasions and to whom I am indebted for much valuable advice concerning the planning of the work.

To the Chief of Forests of *Uddeholm's AB*, Skogschefen G. WESSLÉN, I wish to convey my sincerest thanks. As President of the Board of the *Arbetsfysiologiska Undersökningen* he saw from the beginning the practical importance of the present investigation and it is thanks to his active support that it could be accomplished.

The initiative for the investigation came from the *Society for Värmland's Forest Work Studies (Föreningen Värmlands Skogsarbetsstudier)*, and the work was done in close cooperation with this Society. For all this I am greatly indebted to its Chief, Civilingenieur G. LUTTMAN and its Forest Officer P. OLSSON. I further desire to extend my best thanks to Fil. Kand. G. ALMQVIST of the *Society for Forest Work (Föreningen Skogsarbeten)* who has helped us with the statistics.

To my friends at the experimental Station of *Arbetsfysiologiska Undersökningen*, technical personnel as well as experimental subjects, I wish to express my great appreciation of their good friendship and skilled help.

Finally, my thanks are due to Dr. J. G. STEPHENS, for his kindness in correcting the English text, and to Miss I. LAGERGRÉEN, who designed the diagrams.

The costs of the investigations have been defrayed by grants from *Svenska Arbetsgivareföreningen*, *Svenska Lantarbetsgivareföreningen*, *Föreningen Värmlands Skogsarbetsstudier*, *Föreningen Skogsarbeten* and *Sandvikens Jernverks AB*.

Stockholm, July 1946.

Nils P. V. Lundgren.

Purpose of the Investigation

Since time immemorial forestry work has been done without any very fixed times of work either for the daily hours or for the seasonal schedule. This state of affairs still exists very generally to the extent that the individual worker himself usually determines the days on which he wishes to work, the working hours and the exploitation of these hours. In connection with the attempts to rationalize lumber getting which have been made during the last few years the question arose whether it was not possible to introduce more regulated conditions. Such regulation might be expected to lead to a more rational utilisation of the attenuated number of workers who offer themselves for lumber getting and even to other advantages of a technical, economic and social nature (cf. WESSLÉN 1946).

From the standpoint of quantitative work studies the following questions now arise:

- 1) Can lumber work be continued throughout all seasons of the year?
- 2) How long shall the working day be and how shall it be planned?

In the majority of other trades there exist generally accepted and in many cases legally regulated working hours. As, however, lumber work in comparison with most other trades calls for a very high energy output it has been assumed without exact evidence that one could not apply here the working hours generally accepted industrially. Such hours might possibly be considered to bring about disadvantages in such matters as the workers' health, their frame of mind and their output. These considerations have led The Swedish Committee on Industrial Physiology (Arbetsfysiologiska Undersökningen) to examine the questions of suitable working hours and planning of the working day of lumber getters. This work was carried out in conjunction with its forestry organisa-

tions, The Society for Värmland's Forest Work Studies (Föreningen Värmlands Skogsarbetsstudier) and The Society for Forest Work (Föreningen Skogsarbeten). The investigation was done at the Arbetsfysiologiska Undersökningen's research station at Nilsby, St. Kil's parish, Värmland.

In the present paper the terms lumber work and forestry work include tree-felling, pruning and trimming of felled trees, barking, cross-cutting, cleaving, skidding, yarding or bunching and stacking of timber, pulpwood and firewood.

General Planning of Investigation

Meaning of the term "suitable working hours"

The question of suitable working hours in industrial work may be considered from four principal aspects, namely:

- 1) The subjective fatigue effects and the worker's attitude towards the work. These matters are chiefly psychological and in the present investigation were treated only on very general lines.
- 2) The quantity and quality of the output. These questions as well as others of a technical and economic nature have been treated according to norms which were derived from the investigations carried out by The Society for Värmland's Forest Work Studies (Värmlands Skogsarbetsstudier). The results on this point as well as on the subjective effects will be published separately.
- 3) The physiological effects both immediately and in the long run. This is the principal subject of the present paper.
- 4) The accident risks. This question has been taken into account but the data available are too small to permit any general conclusions.

Social and technical conditions

It is at once obvious that a complete investigation of the optimal conditions in regard to each of the above four aspects of suitable working hours would be an enormous work. For practical reasons it was also apparent at the first planning, that the research would have to be arranged in accordance with the resources available.

Accordingly the procedure was as follows: a fixed time schedule for the working day was arranged which could be regarded as

suitable from general practical quantitative work studies and physiological experience. This schedule was followed as closely as possible by a number of trained lumber getters who were under continuous observation with regard to their output and their subjective and physical reactions.

The time schedule for the working day had been worked out by Värmlands Skogsarbetsstudier. The norms for this were derived partly from experience gained in technical work studies on lumber getting (WESSLÉN, LUTHMAN, OLSSON et al. 1943, LUTHMAN and OLSSON, 1945) and partly from previous physiological investigations on lumber cutting (BOALT and ZOTTERMAN, 1943, LUNDGREN and ZOTTERMAN, 1943). Table 1. gives the time schedules which were proposed for the different seasons, the appropriate total time for the workman's daily presence in the forest and the corresponding actual work time (total time less the time for meals and rest pauses).

The term appropriate time is used to mean the time the workers should keep, weather permitting. When calculating this the conditions of daylight were as far as possible taken into consideration but the times given during the dark period of the year can not be followed very exactly, of course, even with clear weather as it is difficult to define the necessary amount of light for lumber work and also because the time required for the journey to and from the place of work varies very much with the terrain conditions and with the light. The table nevertheless gives an indication of the order of magnitude of these times. It will be seen that the desired work time for whole week days varies between about five hours at the darkest time of the year and seven and a half hours during the brightest.

In conclusion it can be said that when constructing the time schedule there was a general attempt partly to separate the working periods by pauses sufficiently frequent and long as well as by meal periods, and partly to attain a moderate and uniform work intensity by spreading the effort over a fairly long total time. Through this planning a routine was sought which would give an even working tempo with sufficient recovery times to yield an effective day's work.

Because of the long rest and pause times the workers had to stay

TABLE 1. *Time schedule for the working day in the forest during different seasons, drawn up by the Society for Värmland's Forest Work Studies. The real work time consists of the total time in the forest exclusive of the time for meals and rests, the latter being calculated as 7 minutes per hour of work.*

Period	Time of day	Schedule	Minutes	Daily total time in the forest, hours	Daily real work time, hours
1/1—15/1 Whole weekdays	8.15— 9.45	Work period I	90	7.5	5.3
	9.45—10.15	Breakfast	30		
	10.15—12.00	Work period II	105		
	12.00—12.45	Lunch	45		
	12.45—14.15	Work period III	90		
	14.15—14.30	Coffee	15		
	14.30—15.45	Work period IV	75		
Saturdays	8.15— 9.45	Work period I	90	5.75	4.4
	9.45—10.15	Breakfast	30		
	10.15—12.00	Work period II	105		
	12.00—12.15	Coffee	15		
	12.15—14.00	Work period III	105		
16/1—31/1 Whole weekdays	7.45— 9.30	Work period I	105	8.5	6.2
	9.30—10.00	Breakfast	30		
	10.00—12.00	Work period II	120		
	12.00—12.45	Lunch	45		
	12.45—14.30	Work period III	105		
	14.30—14.45	Coffee	15		
	14.45—16.15	Work period IV	90		
Saturdays	7.45— 9.30	Work period I	105	6.25	4.9
	9.30—10.00	Breakfast	30		
	10.00—12.00	Work period II	120		
	12.00—12.15	Coffee	15		
	12.15—14.00	Work period III	105		
1/2—15/2 Whole weekdays	7.15— 9.15	Work period I	120		
	9.15— 9.45	Breakfast	30		
	9.45—12.00	Work period II	135		

Period	Time of day	Schedule	Minutes	Daily total time in the forest, hours	Daily real work time, hours
Saturdays	12.00—12.45	Lunch	45	9.5	7.1
	12.45—14.45	Work period III	120		
	14.45—15.00	Coffee	15		
	15.00—16.45	Work period IV	105		
	7.15— 9.00	Work period I	105	6.25	4.9
	9.00— 9.30	Breakfast	30		
	9.30—11.30	Work period II	120		
	11.30—11.45	Coffee	15		
	11.45—13.30	Work period III	105		
16/2—31/10 Whole weekdays	7.00— 9.00	Work period I	120	10.0	7.5
	9.00— 9.30	Breakfast	30		
	9.30—12.00	Work period II	150		
	12.00—12.45	Lunch	45		
	12.45—14.45	Work period III	120	10.0	7.5
	14.45—15.00	Coffee	15		
	15.00—17.00	Work period IV	120		
	7.00— 8.45	Work period I	105		
	8.45— 9.15	Breakfast	30		
	9.15—11.15	Work period II	120		
Saturdays	11.15—11.30	Coffee	15	6.25	4.9
	11.30—13.15	Work period III	105		
	7.15— 9.00	Work period I	105		
	9.00— 9.30	Breakfast	30		
1/11—15/11 Whole weekdays	9.30—12.00	Work period II	150	8.75	6.4
	12.00—12.45	Lunch	45		
	12.45—14.15	Work period III	90		
	14.15—14.30	Coffee	15		
	14.30—16.00	Work period IV	90	8.75	6.4
	7.15— 9.00	Work period I	105		
	9.00— 9.30	Breakfast	30		
	9.30—11.30	Work period II	120		
	11.30—11.45	Coffee	15		
	11.45—13.30	Work period III	105		

Period	Time of day	Schedule	Minutes	Daily total time in the forest, hours	Daily real work time, hours
16/11—30/11 Whole weekdays	7.45—9.15	Work period I	90	8.0	5.7
	9.15—9.45	Breakfast	30		
	9.45—12.00	Work period II	135		
	12.00—12.45	Lunch	45		
	12.45—14.15	Work period III	90		
	14.15—14.30	Coffee	15		
	14.30—15.45	Work period IV	75		
Saturdays	7.45—9.30	Work period I	105	6.25	4.9
	9.30—10.00	Breakfast	30		
	10.00—12.00	Work period II	120		
	12.00—12.15	Coffee	15		
	12.15—14.00	Work period III	105		
1/12—31/12 Whole weekdays	8.15—9.45	Work period I	90	7.25	5.1
	9.45—10.15	Breakfast	30		
	10.15—12.00	Work period II	105		
	12.00—12.45	Lunch	45		
	12.45—14.00	Work period III	75		
	14.00—14.15	Coffee	15		
	14.15—15.30	Work period IV	75		
Saturdays	8.15—9.45	Work period I	90	5.75	4.4
	9.45—10.15	Breakfast	30		
	10.15—12.00	Work period II	105		
	12.00—12.15	Coffee	15		
	12.15—14.00	Work period III	105		

quite long in the forest as their places of work were generally so distant from their homes that they could not go home for meals during working hours. The disadvantage of this has been counteracted by a series personnel welfare arrangements. Among these may be mentioned the portable rest-huts of Värmland's Skogsarbetsstudier at the working places, these huts being supplied with

handy and effective heating arrangements. Further, suitable clothing for bad weather was tried out by Trelleborgs Gummi-fabriks AB in conjunction with the above institution. Further details on these matters are given by LUTHMAN and OLSSON (1945).

Where fairly large distances were involved and where road conditions were bad the men were taken by motor-car to and from work. Efforts were also directed towards providing as good facilities as possible for recreation during the men's spare time. Among other things the care and reconditioning of the working tools which otherwise occupies time in the evenings and on Sundays were taken over by the service workshop, which has been run for several years at Munkfors in Värmland by Värmlands Skogsarbetsstudier. In this way tools were exchanged according to a fixed routine which took into account the varying time at which they normally wear out. Arrangements here were flexible, however, and tools were exchanged at other times when expedient.

The subjects of these experiments were encamped in lumber workers' barracks of the Swedish State Labour Commission, and the common household was conducted by trained women cooks. The investigation took place during the years 1944 and 1945 and thus during a period of food rationing. As, however, free accessibility to all foods is an important factor in the efficiency of output in a trade involving very heavy labour, it was desirable to avoid the limitations of rationing. This was made possible by the State Food Commission which, on the instigation of Professor ZOTTERMAN, very kindly placed extra licenses at our disposal. These were sufficient for free choice of food, thus providing a good peacetime standard.

At repeated intervals the food consumption of each subject was determined. As an example it may be mentioned that one of these investigations showed an average daily consumption of each subject of 231 gm fat, 757 gm carbohydrate, 185 gm protein, 3.0 gm Ca, 4.1 gm P, 33 mg Fe, 5.800 I. U. vitamin A, 3.3 mg vitamin B₁ and 95 mg vitamin C. The complete results will be published elsewhere.

The workmen concerned were under contract and were paid in the following way. For all normal work they were paid according to the local collective agreement. For the time spent in physiological

experiments they received payment on a time basis which was calculated from their worktime earning in the forest during the present period of two weeks, together with 10 % extra for their trouble. Worktime earnings were determined from continuously conducted statistics on their earnings and working times. Finally, each subject received a "long service reward" of 250 Swedish Kr. which was paid out at the end of contract and which was regarded as a compensation for personal trouble associated with their somewhat long absences from home.

This outline will be given in more detail in a comprehensive account of the physiological research applied to lumber getting which is to be published separately.

Data concerning workmen used as experimental subjects

The very detailed study of each subject which was considered necessary, together with all other data concerning their daily output which the research station had to collect, limited the number of subjects investigated to five. This number may seem too low for any general conclusion to be drawn concerning the suitability of the time scheduled work in this field. An attempt to counteract this limitation has however been made by the selection of subjects. Trained lumber workers were sought, who might be considered to represent constitutionally average types and whose previous output was on an average level. An age distribution was selected to give representation even of the higher ages.

Table 2. gives some data concerning the experimental personnel. It is seen that their ages at the outset were 26, 28, 40, 41 and 55 years. The experiments were aimed to deal with trained lumber getters and accordingly the following notes on their personal working life are given.

E. L. Started as a farm hand at the age of 14. From his 22nd. to his 25th. year he was a lumber worker in the winter and a railroad track worker in the summer. After this he was lumber worker the

TABLE 2. *Data concerning the subjects at the outset of the investigation.*

Subject	Born	Age years	Observation time		Body height Cm.	Body weight Kg.	Vital capa- city Litres
			Dates	Number of months			
E.L.	11.12.89	55	14.3— 20.12.45	9	167.5	65.6	5.08
J.O.	9. 4.03	41	17.10.44— 20.12.45	14	170	73.0	5.31
J.S.	7. 3.04	40	14.11.44— 20.12.45	13	180.5	74.2	4.54
B.N.	10. 9.16	28	3.10.44— 20.12.45	14 ¹ / ₂	176	66.2	5.47
O.F.	1. 6.18	26	15.2 — 20.12.45	10	184.5	71.8	6.34

whole year round until 37 years of age. He had his own farm from his 37th. to his 46th. year, did lumber work except for short work periods on his own farm until his 53d. year after which he was engaged exclusively on lumber work. He used to take at most ten days vacation per year. No military service during recent years.

J. O. Began working at the age of 14. Tree marking in the forest first year. Blacksmithing from 15 to 24 and horseshoeing from 32 to 34. Otherwise lumber work and running his own farm of about 3 acres. After 1939, he spent 240 days in military service in several periods. He never had a real holiday and only in occasional years could he take a few days off work as agriculture took all his time when he was not working in the forest.

J. S. Started work at 14 in semi-heavy industrial work, rolling mill and screw factory during 3 years. He then worked at charcoal burning, road and railroad building and lumber work until 29 years of age. Between 29 and 35, heavy outdoor industrial work. After

this, lumber work with periods of agricultural work which latter amounted to one year. No military service.

B. N. Started on the farm at 14 helping his father who was a carpenter. After the age of 17, forest work, lumber getting as well as horsediving interspersed with agricultural work and shorter periods of carpentering and gardening. About $1\frac{1}{2}$ years military service in several periods. Has studied mathematics and technical subjects quite extensively during spare time by correspondance.

O. F. Started work at 13 as an errand-boy. After 3 years of this he worked in a rolling mill for 5 years. Lumber worker since the age of 21 interrupted only by short periods of road work. About $1\frac{1}{2}$ years military service in several periods.

Total observation time and its bearing on the validity of the conclusions

As Table 2. shows, the period during which the experimental subjects were observed varied between approximately 9 and $14\frac{1}{2}$ months. The times stated refer to continuous work of all varieties which occur in the natural sequence of lumber work throughout the year (cutting of timber, pulpwood and firewood). The periods are indeed not so long that it is permissible to draw conclusions as to the suitability of the time scheduled work for really prolonged times. Any investigation which would enable this to be done would have to be on quite another scale. In the first place it would be necessary to consider statistically large groups working for long periods according to the norms given above or otherwise well defined norms. It may be added that such material is not obtainable at present because there does not exist any sufficiently accurate record of working hours in forestry work. Moreover, the current system of payment does not adequately reflect the amount of labour involved in a given piece of work. Again, it is difficult to obtain workers who are lumber workers only, because forestry work is essentially seasonal and is intermixed with phases of agricultural work etc.

In regard to these limitations of the time of observation it must therefore be stated here that the questions to which the investigation may be expected to answer, are mainly:

- 1) The practical possibility of applying an arranged working time schedule when external conditions are favourable.
- 2) The associated earning possibilities.
- 3) The lumberman's own opinion as to the suitability of the schedule.
- 4) The effect of the work upon bodily function during the course of the day, the week and the various cutting periods of the year.

Among these questions it is only the latter which will be treated in this paper, the remaining topics will be dealt with elsewhere.

Energy output during lumber work in the light of previous investigations

When investigating the effects of industrial work it is first necessary to inquire in which respect it places demands upon the body. With knowledge of the bodily functions specially concerned it is then possible to gain an insight into the degree of adaptation or overloading, should such occur.

It has long been recognised that lumber work calls for a large energy output. TIGERSTEDT (1900) showed, in an examination of 96 lumber workers from Ångermanland in north Sweden, an average daily consumption of 6.231 Kcal. calculated from non-prepared foodstuffs. WOODS and MANSFIELD (1904) in an investigation made in Maine found a daily supply of 8.140 Kcal. brutto. BOALT and ZOTTERMAN (1943) as well as LUNDGREN and ZOTTERMAN (1946) report, from individual food investigations during the years 1941 to 1945, averages of the daily net energy supply of 4.548 to 5.914 Kcal. (Table 3). It may also be mentioned here that LUNDGREN and ZOTTERMAN (1943) using another method, the estimation of the energy metabolism per minute during the different working operations together with time studies, calculated a metabolism of 5.700 Kcal for 24 hours.

TABLE 3. *The daily energy output in lumber work derived from individual food-investigations.*

Author	Place	Time of investigation	Number of subjects	Kcal. per 24 hours	Real work time per day in hours	Occupation
Boalt and Zotterman (1943)	Värmland	March to April 1942	19	5.914±370	—	Timber-cutting
	Värmland and Dalarna	Dec. 1942 to Jan. 1943	16	4.548±220	—	"
Lundgren and Zotterman (1946)	Värmland	Nov. 1942	5	4.653±149	5.7	"
	"	April 1944	4	5.896±225	7.0	"
	"	March to April 1945	7	5.869±285	6.8	"
	"	Aug. 1945	5	5.204±297	6.2	Cutting of fire-wood and pulpwood

As regards the energy output during the different working operations in the forest the investigations of LUNDGREN and ZOTTERMAN (1943 and 1946) may be cited. The results of these are summarised in Table 4., which comprises both winter and summer periods, although during the summer only two subjects were examined. These two persons were however specially selected being types at opposite extremes. Thus the mean value may have some significance. From Table 4. it emerges that the energy output is high at practically all the working operations which were studied, and these represent the major part of all varieties of lumber work.

TABLE 4. *The energy output for different types of forest work, according to Lundgren and Zotterman (1943 and 1946).*

Type of work	Number of subjects	Kcal./min.	
		Mean	Range
<i>Timber cutting (in the winter)</i>			
Tree-felling with felling-saw	11	10.7	8.4—12.7
Trimming of felled trees	11	10.2	8.7—11.6
Barking	11	10.1	8.5—12.0
Cross-cutting with bucksaw	11	9.0	7.5—10.5
<i>Fire-wood cutting (in the summer)</i>			
Tree-felling with bucksaw	2	9.6	8.2—11.0
Trimming of felled trees	2	8.4	8.1— 8.6
Dragging of firewood	2	9.8	8.7—10.9
Cross-cutting in a sawhorse	2	7.8	6.9— 8.7
Barking in strips	2	5.9	5.2— 6.5
Cleaving of softwood	2	9.7	9.2—10.1
Cleaving of birch billets	2	8.9	8.6— 9.1
Stacking of firewood	2	6.3	5.7— 6.9

Planning of the investigation with regard to the measurement of the bodily functions

The continuous examinations of the bodily state of the experimental personnel was aimed to ascertain whether any deterioration occurred in the course of the experimental period which could be attributed to the work or the method of working. The investigations were partly clinical bodily examinations and partly functional tests.

The functioning systems which might first be expected to be exposed to overloading during exceptionally heavy work are the neuro-muscular apparatus and the oxygen transport mechanism. It has been assumed that the clinical control could be relied upon to detect any sign of overload upon the neuro-muscular apparatus, while the specific functional tests were directed towards the oxygen transport apparatus.

Any changes, whether for better or worse, shown by the functional tests could naturally be interpreted in several ways. A change indicating improved function of a healthy adult who lives under relatively permanent hygienic conditions is to be interpreted as showing that the work has had a greater training effect than the person's previous occupation, so that there has occurred an adaptation of the oxygen transport apparatus to the heavier work. A change for the worse on the contrary may imply either that the work has become lighter and therefore has had less training effect, or that illness or other damaging agents have come into operation, or finally that the work has been so heavy that an overstrain has occurred.

Because of the necessity of taking into account external factors other than the work itself, it was essential to record as far as possible all conceivable agencies even outside the work. As a part of this general control daily entries were made of any signs of chill or other illness, or reports about such, together with any circumstances which might influence the measurements. As an example, records were made when the experimental subjects went to bed late, whether they had attended a dance or sat up late reading or taking part in athletic sports the previous evening, when they had got up in the night to smoke, slept badly, had been awake before the morning examination etc. This very careful recording was only possible for the reason that the experimental subjects lived in one household whilst the examiners lived in continuous intimate contact with them even in the spare-time.

The tests applied to determine the function of the oxygen transport system were:

- 1) Tests to estimate the functional state from day to day and week to week during the total investigation period:
 - a) Resting tests: morning pulse-rate and blood pressure whilst lying in bed fasting.
 - b) Exercise tests: Schneider index and bicycle ergometer tests.
- 2) Tests aimed to estimate the function during the course of the working day: exercise tests morning and evening on the bicycle ergometer and while cross-cutting.

Medical Examination

Methods

The medical examination of each subject consisted of

I. Anamnesis, physical examination including examination of the nervous system, determination of the vital capacity of the lungs and the haemoglobin content of the blood, as well as urine tests for protein and sugar at the beginning and at the end of the total period.

II. Daily notes during the course of the observation period.

III. Electrocardiography and radiography of heart and lungs on repeated occasions.

IV. Frequent determinations of body-weight and rectal temperature in the morning on rising.

Medical history notes and physical examination: These were recorded in the usual way. Special attention was given to any factors which might indicate any deleterious influence of the work either before or during the period of observation. Thus all signs which might have any bearing upon the functioning of the respiratory, circulatory, digestive or motor systems were kept in closest view.

The examination of the nervous system involved assessment of the psyche, cranial nerves, motility, reflexes, coordination and sense organs.

The vital capacity of the lungs was estimated in the standing position using KROGH's spirometer, several measurements being made each time and the highest value taken as the vital capacity. The subjects had rested before the measurements.

According to HUTCHINSON, DREYER and others, the vital capacity stands in a certain relation to the body-weight, height and body

surface, and of these the closest correlation is that with the body surface (cit. BEST and TAYLOR 1943). WEST (1920) states that the vital capacity in physically well trained persons averages 2.8 litres per square meter of body surface. In the present study the "standard values" as above have been calculated for each subject and compared with those actually found. The body surface was calculated from the nomogram derived from MEEH's formula as modified by DU BOIS and DU BOIS.

Blood Haemoglobin was estimated with the Sicca haemometer. This method is shown by EHRENBERG (1943) to be the best ordinary clinical method. The haemometer used was calibrated against an accurately controlled AUTENRITH haemometer. Each result given represents the mean of 15 to 20 separate readings, the standard error of the mean amounting to ± 0.3 to ± 0.5 per cent of the obtained value.

Urine examination was limited to the ALMÉN's and NYLANDER's test for glucosuria and HELLER's reaction for proteinuria. Tests were made on morning urine as well as during the day.

Electrocardiography was done at intervals of some months in the medical department of the county hospital in Karlstad¹). The examination was done according to usual clinical norms and consisted of resting records with leads from the extremities and chest as well as records from the extremities after running upstairs.

Radiography of the heart and lungs was undertaken in the radiological department of the county hospital in Karlstad¹).

Body-weight was at first measured several times only during the month but later almost every day. The weighing was done in the morning immediately after rising and passing urine. It was made by a Stathmos balance and after instruction the men were able to use it themselves. For calculating the error inherent in the method a series of double weighings were made by each person on different days. Between these measurements the balance was returned to zero position. The men themselves adjusted the instrument exactly to

¹) In this connection we wish to extend our heartiest thanks to the head physicians Dr. J. Svensson and Dr. J. Andrén for their kind help.

zero before each weighing. From these double determinations the experimental error was calculated according to the formula (DAHLBERG 1940)

$$\sigma_{\text{method}} = \frac{1}{\sqrt{2}} \cdot \sigma (\Delta)$$

where Δ is the difference between the first and the second readings.

These errors were:

for E. L. ± 0.08 kg., J. O. ± 0.08 kg., J. S. ± 0.10 kg., B. N. ± 0.03 kg., O. F. ± 0.03 kg., which show that the weighings were sufficiently accurate.

The average error for all was ± 0.07 kg.

Rectal temperature was measured with a clinical thermometer of the usual type, the reading being made after at least 3 minutes. Each thermometer used was calibrated against a control thermometer of aged glass. The experimental errors were estimated from a series of 49 double measurements before which the subject had been lying at least half an hour in bed and had reached a temperature equilibrium. The experimental error amounted to ± 0.03 C.

Results

Case history notes, physical examination and state of nervous system: None of the subjects showed any noteworthy signs or symptoms at the beginning nor did any such appear at the end of the observation period. It seems unnecessary therefore to give any full account of the data collected in this respect. The deviation of the basal metabolism determined with KROGH's apparatus from the calculated normal value for all experimental subjects lay within the limits $+ 15$ to $- 5$ per cent.

Vital capacity of the lungs: The results here are shown in Table 5 which gives the observed values, calculated standard values according to WEST, together with the percentual deviations from these. For comparison, measurements on other trained lumber workers who worked at our research station are included in the table. It

TABLE 5. Calculated body surface area, calculated vital capacity of the lungs and recorded vital capacity and haemoglobin content of the blood at the beginning (I) and the end (II) of the observation time.

Subject	Calculated body surface area	Vital capacity				Haemoglobin content %	
		Calculated I.	Recorded I.		Devia- tion %	I	II
			I	II			
E.L.	1.74	4.9	5.1	5.0	+ 4	101	102
J.O.	1.83	5.1	5.3	5.4	+ 6	103	105
J.S.	1.92	5.4	4.5	4.6	-15	103	97
B.N.	1.80	5.0	5.5	5.6	+12	106	104
O.F.	1.93	5.4	6.3	6.4	+19	101	106
No 1	1.85	5.2	6.2		+19		
2	1.74	4.9	5.1		+ 4		
3	1.86	5.2	5.8		+12		
4	1.86	5.2	5.4		+ 4		
5	1.82	5.1	4.2		-18		
6	1.96	5.5	5.9		+ 7		
7	1.92	5.4	5.0		- 7		
8	1.82	5.1	5.5		+ 8		
9	1.93	5.4	5.8		+ 7		
10	2.14	6.0	7.4		+23		

will be seen that the vital capacities of the five subjects who participated in the long-time investigation were practically unchanged at the end. As regards the pronounced minus variants in the table, subject No. 5 had previously suffered from a pleuritis which may explain the low value. This man was otherwise in very good physical condition. The low value of the man J. S. may be explained by the difficulty in getting him to breathe maximally. Among the pronounced plus variants, O. F. was a fairly good runner, No. 1 a very good skier and No. 10 was the subject whose earnings in the forest were higher than those of any other man.

The haemoglobin content at the beginning of the observation period and at its end is given in Table 5. It will be seen that this con-

tinued practically unchanged. J. S. showed a slight reduction at the end of the period which was found to be permanent. This reduction may be related to a chill which he suffered. The value nevertheless lay within normal limits.

Urine examinations. None of the men showed glycosuria but O. F. showed proteinuria on several occasions in repeated tests during work as well as when resting in an upright position. After resting in bed the HELLER test was negative. Urinary sediments were entirely normal and there was no history of renal damage. It is therefore likely that this represented an orthostatic or functional proteinuria. No further examinations were done to clarify this matter nor were they called for, but the diagnosis is nevertheless given with reservation.

Electrocardiographic records were done for each subject at the beginning and the end of the observation period and further at three times in between. For practical reasons the records could not be done under fully controlled conditions and it must therefore be stated quite clearly that the value of these particular investigations is rather limited. As far as ordinary clinical tests enable the question to be answered, however, in no case did any pathological changes occur. All records varied within the normal range¹).

X-ray examination of heart and lungs was done five times for each man during the course of the observation. The lung findings were all normal. The same applies to the shape of the heart and the aorta and their pulsations.

Heart volume measurements were made orthodiagraphically as well as by measuring antero-posterior radiographs. As, however, the measurements on the different occasions were made by different clinicians and as the method was not exactly fixed the examination does not claim to give any very accurate information about the changes of the heart volume during the experimental period. A more accurate method would, of course, have been extremely desirable for the evaluation of the effect of the time schedule work upon the heart, but unfortunately could not be done for practical reasons. The value of the investigation is thus limited to the

¹) The records have been investigated by doctor *Gunnar Malmström*, The Serafimer Hospital, Stockholm, to whom I wish to convey my best thanks.

TABLE 6. *The heart volume and the largest transversal diameter of the heart. Averages of five records of each subject at different times.*

Subject	Transverse measurement cm.			Volume of the heart ml.		
	To the right	To the left	Total	Total	per m ² of body surface area	per kg. body weight
E.L.	5.0	8.8	15.8	975	540	14.4
J.O.	3.9	9.8	13.7	820	450	11.5
J.S.	4.6	8.8	13.4	730	360	9.5
B.N.	4.7	8.3	13.0	730	390	10.7
O.F.	4.7	8.8	13.5	720	370	9.9

extent that, although the repeated measurements give general information about the order of magnitude of the heart volume of the men, they nevertheless tell nothing about any changes. For this reason it seems more suitable to mention only the average of the several values for each subject (Table 6). It is seen that E. L. manifestly exceeds the upper normal limit, 450 ml, per square meter body surface, stated by JONSELL (1939). J. O. reaches up to this limit, while the values for the others lie entirely within the normal limits.

The body-weight varied during the observations as is shown in Fig. 1, which gives the average monthly weights for each man. It was found unnecessary to treat the material statistically as no further information could thus be gained. It will be seen that O. F., J. O. and B. N. all show variations within narrow limits throughout, while E. L. shows a moderate and J. S. a large increase in weight (about 6 kg.). The increase in weight of the latter two may be related to the fact that these men, before their engagement at the research station, lived alone, and did their own housework and cooking under rather primitive conditions, whilst the others lived with their families. The subject B. N. showed a loss of weight

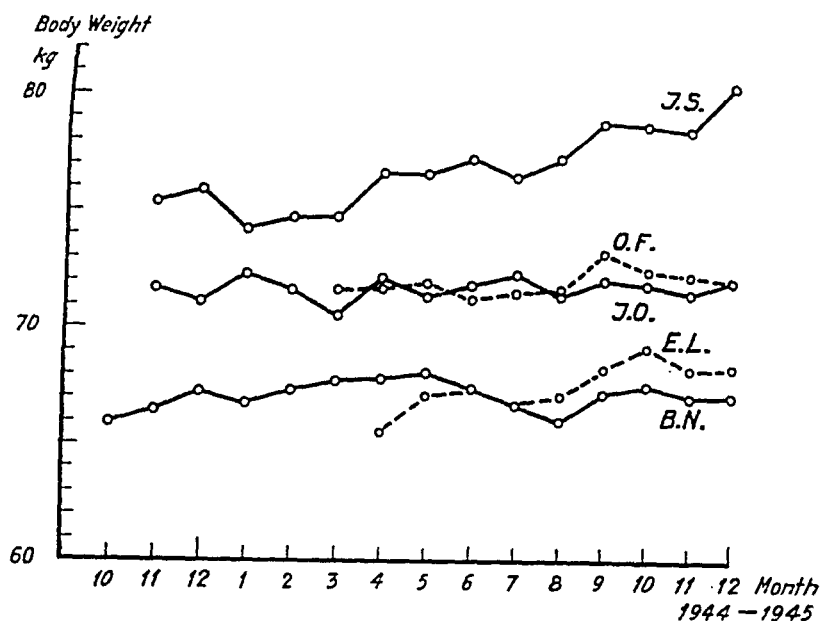


Fig. 1. Body weights, averages per month.

of gradual onset during the summer months while the others during this season were practically unchanged in weight.

The rectal temperature in the morning before rising was, at the beginning, recorded only when the men were chilled and on days

TABLE 7. Rectal temperature in the morning, before rising, empty stomach. Averages per month, °C.

Month 1945	Subject				
	E.L.	J.O.	J.S.	B.N.	O.F.
March		36.27	36.38	36.51	36.48
July	36.57	36.62	36.55	36.40	36.48
August	36.49	36.46	36.58	36.46	36.46
September	36.43	36.56	36.68	36.44	36.38
October	36.50	36.57	36.65	36.39	36.38
November	36.53	36.57	36.66	36.40	36.48
December	36.58	36.59	36.74	36.41	36.56

when the temperature was recorded during work. Only during the later months were daily measurements done. Because of this scanty material only the average values of the daily temperature during these months are given, when a large number of measurements were done independently of the state of health (Table 7). Statistical treatment with regard to the monthly variation has not been done for the reason that nothing further would thus be disclosed. The table shows that the averages values vary only over a very small range. These averages afford no basis for separation of summer from winter temperatures. Nor could such a separation be expected from the previous results of BURTON, SCOTT, Mc GLONE and BAZETT (1940).

Accidents during work were rare except for smaller bruises which the men themselves could treat and which did not affect their working capacity. E. L., however, suffered a saw-injury to his left hand which involved three days absence from work and J. O. damaged his left fore-arm from a recoiling wedge and was also absent from work three days.

Resting Pulse-Rate

Earlier Investigations

It is a well known fact that persons in good physical training often exhibit bradycardia. (BUCHANAN 1909, COOK and PEMBREY 1913, HENDERSON, HAGGARD and DOLLEY 1927, HOOGWERF 1929, BRAMWELL and ELLIS 1929, COTTON 1932, HERXHEIMER 1933, BORGARD 1937, WHITE 1942). Furthermore, Mc CURDY (1910) and MEYLAN (1913) consider that the resting pulse-rate in prone and standing positions is of significance in the estimation of the subject's physical condition. BROUHA and HEATH (1943), in investigations on students, could nevertheless not find any relation between the resting pulse-rate in prone or sitting positions and the capacity to perform hard muscular work.

Slowing of the pulse-rate during physical training has been demonstrated by MICHELL (1909), DAWSON (1919), SCHNEIDER, CLARK and RING (1927), HOHWÜ CHRISTENSEN (1931), SCHNEIDER and CRAMPTON (1940), and also by KNEHR, DILL and NEUFELD (1942). The latter found that the pulse-rate of 14 students who trained systematically for six months, was slowed by 5 beats per minute, and this was the only change which could be demonstrated in the resting functions. BROUHA and HEATH (1943), found that the sitting pulse-rate of university oarsmen was slightly lower after training.

The resting pulse-rate is of course affected by a variety of factors other than the state of physical training. Among these are a number of pathological conditions and psychical influences as has been demonstrated by BROUHA and HEATH (1943). According to HENRY (1942) deprivation of sleep for 24 hours results in a lowering of the heart rate in the reclining position but does not affect the rate in a standing position. As far as seasonal varia-

tions are concerned GUSTAFSSON and BENEDICT (1928) found minimal pulse-rates in January, but GRIFFITH, PUCHER, BROWNELL, KLEIN and CARMER (1929) observed, on the contrary, that the summer rates were minimal. According to the latter authors "such a state of affairs must preclude all thought of a direct climatic effect and must direct the search for the cause of these variations into a closer scrutiny of the daily habits and activities of the subjects".

HOHWÜ CHRISTENSEN (1939) maintains that the changes in the resting pulse-rate in the same individual during athletic training give a good indication of the functional condition of the oxygen transport apparatus. It is of course manifest that among different individuals there are variations which arise not only from the functional state; but as the present experiments are not concerned with the comparison of different persons but to follow the developments in the same person, it was considered that the results of resting pulse-rate determinations could be regarded as significant.

Methods

On all the experimental subjects the radial pulse-rate was counted practically every week-day morning before the subject arose from bed or ate. As a rule the men were sleeping until immediately before their pulse-rate was taken. If it so happened that a man had got up for some reason or other before his pulse was recorded he was made to lie down again for at least 30 minutes before the measurement, which period is more than sufficient, according to COLLETT and LILJESTRAND (1924), for the pulse-rate to return to a basal level.

Pulse-rate determinations were made with a stop-watch, the number of beats per half-minute being recorded. Determinations were continued until a constant level was reached, whereupon the lowest reading was taken as the day's value.

Results

Normal days: Results of the resting pulse-rate determinations are grouped in averages for periods which ranged from 3 to 5 weeks

TABLE 8. *The morning pulse-rate in recumbent position before food intake. The means and their standard errors for periods of normal days.*

Period No	Date	Subject				
		E.L.	J.O.	J.S.	B.N.	O.F.
	1944					
1	2.10—21.10				53.2±0.5	
2	23.10—18.11		49.4±0.3		52.0±0.5	
3	20.11—20.12		46.3±0.6	52.7±0.4	48.4±0.5	
	1945					
4	8.1—17.2		45.2±0.4	51.8±0.4	50.0±0.5	
5	19.2—23.3		45.4±0.4	52.8±0.4	48.2±0.4	50.8±0.5
6	3.4—28.4	48.7±0.6	45.4±0.5	53.2±0.3	47.1±0.6	51.1±0.5
7	30.4—26.5	47.3±0.8	43.9±0.5	52.4±0.5	46.5±0.6	50.0±0.7
8	28.5—30.6	47.2±0.7	45.0±0.4	52.7±0.3	45.1±0.4	50.0±0.7
9	2.7—4.8	44.8±0.4	46.4±0.6		45.3±0.6	49.4±0.7
10	6.8—1.9	46.5±0.5	44.7±0.3	52.0±0.5	46.7±0.4	50.3±0.4
11	3.9—29.9	46.0±0.4	45.9±0.5	52.9±0.7	46.0±0.5	49.3±0.7
12	1.10—27.10	45.0±0.6	44.9±0.5	52.4±0.5	44.3±0.5	48.0±0.6
13	29.10—24.11	43.4±0.6	44.5±0.4	51.1±0.5	44.5±0.4	47.3±0.7
14	26.11—20.12	44.6±0.5	45.1±0.3		45.1±0.4	47.4±0.4

terminating at a week end. Exactly the same time period could not always be employed because of the interruption of holidays and other matters which necessitated several days cessation of work in the forest, and which were taken to form the end of the period.

As the aim was primarily to determine the effects of the work itself, in the calculation of the averages the values were excluded for days when the journal entries disclosed some factor outside the work which might be considered to affect the functional state. This exclusion was only done with regard to matters of personal history and clinical signs, but was done irrespective of whether any effect on the resting pulse-rate or any of the other functional tests, could be detected or not. Results from such "not normal" days have been collected in a special group for separate evaluation.

Table 8 gives the means for the various time periods of normal

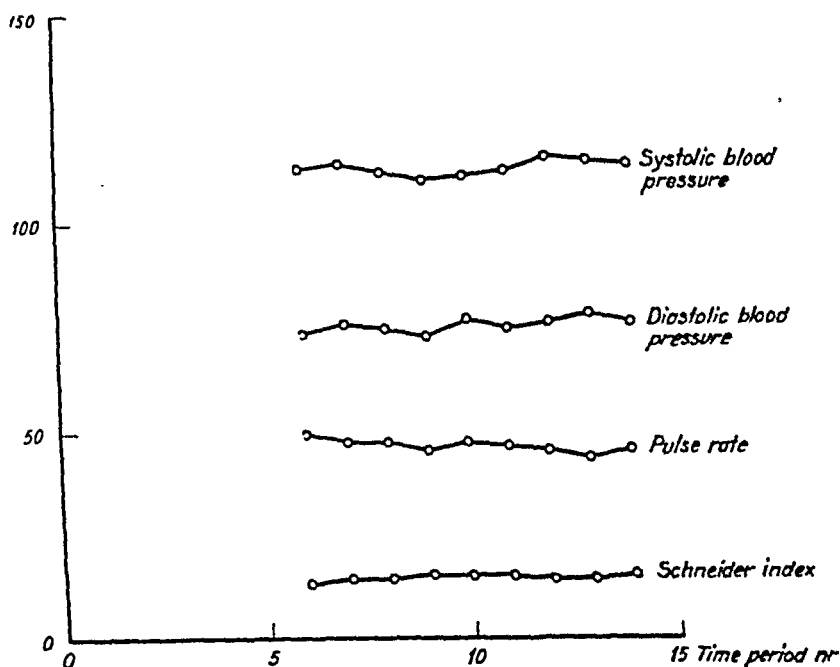


Fig. 2. Morning pulse-rate and arterial blood pressure in recumbent position, and Schneider index during the course of the investigation. Subject E.L.

days, together with the standard errors of the means, As far as the standard deviations for single determinations are concerned, it may be mentioned that they varied between 1.3 and 3.4. The means are also represented diagrammatically in Fig. 2 to 6. It is seen that even at the beginning of the investigation all of the subjects had a low pulse-rate varying from 49 in one man to 53 in another. It should also be mentioned that the lowest daily value encountered in the entire investigation was 39 (subject J. O.). The averages show that the pulse-rates of subjects E. L., B. N. and O. F., had mainly a tendency to fall during the course of the investigation, whilst for J. S. during the whole observation time and for J. O. after a fall during the first period, the rates fluctuated around a fairly uniform level. In order to determine whether the variations between the periods were systematic or incidental, analysis of variance according to the method of FISHER-

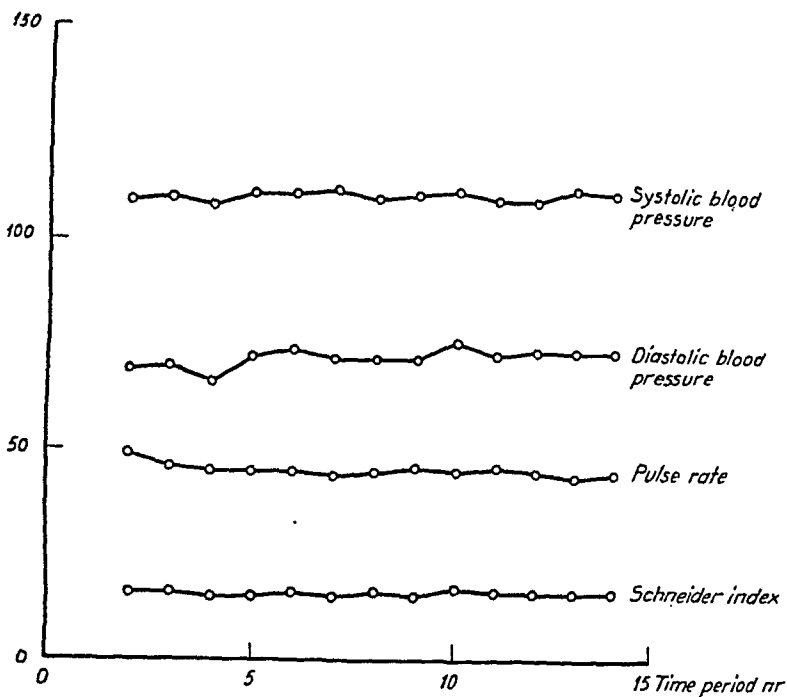


Fig. 3. Morning pulse-rate and arterial blood pressure in recumbent position, and Schneider index during the course of the investigation. Subject J.O.

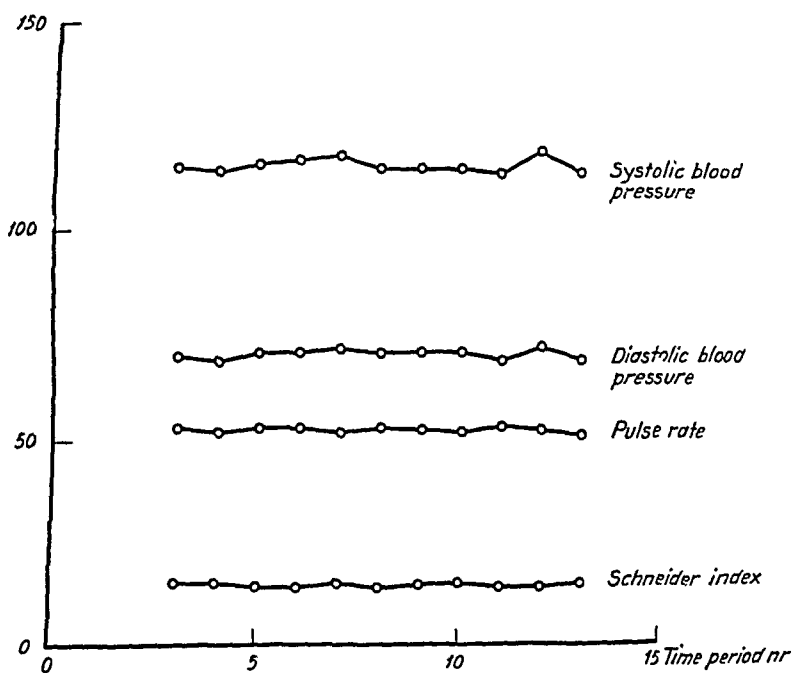


Fig. 4. Morning pulse-rate and arterial blood pressure in recumbent position, and Schneider index during the course of the investigation. Subject J.S.

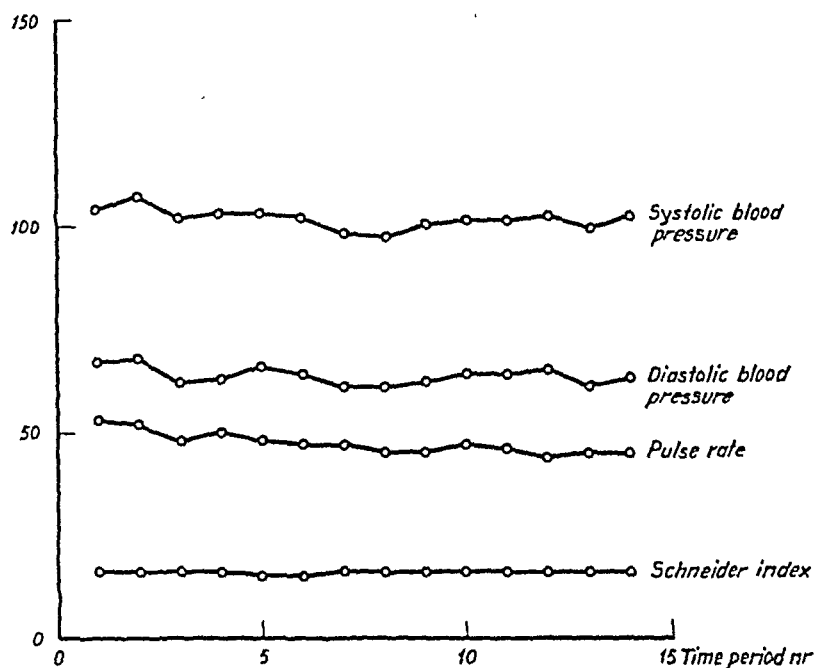


Fig. 5. Morning pulse-rate and arterial blood pressure in recumbent position, and Schneider index during the course of the investigation. Subject B.N.

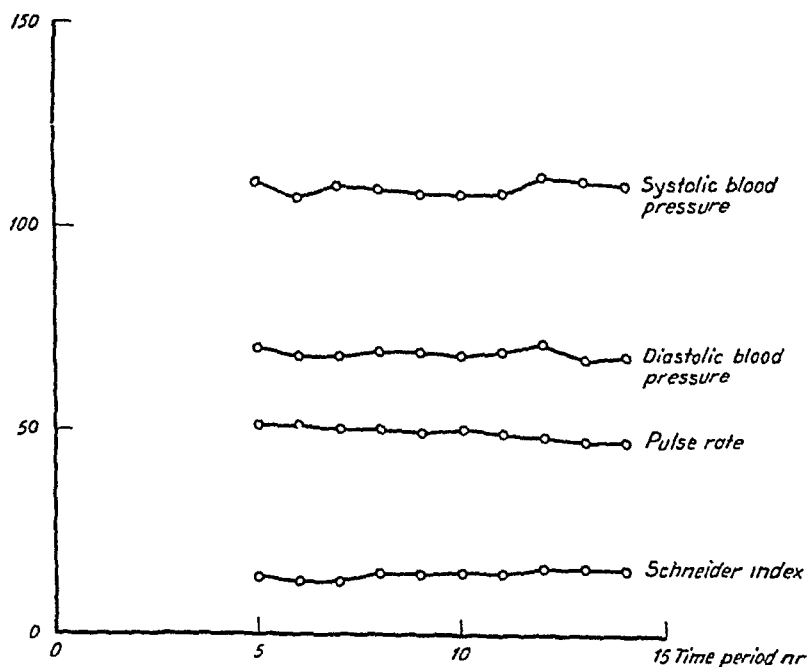


Fig. 6. Morning pulse-rate and arterial blood pressure in recumbent position, and Schneider index during the course of the investigation. Subject O.F.

TABLE 9. *Analysis of variance of the morning pulse-rate in recumbent position within and between periods of normal days.*

Subject	Degrees of freedom		Quotient of variances	P
	1	2		
E.L.	8	142	8.81	<0.001
J.O.	12	231	11.28	<0.001
J.S.	9	205	2.04	0.05—0.01
B.N.	13	268	27.31	<0.001
O.F.	9	170	4.95	<0.001

SNEDECOR was applied. (Table 9). It thus appears that differences between the time periods for four of the experimental subjects were significant and for J. S. probable¹). .

The division between normal and "not-normal" days must, to a certain degree, always rest upon a subjective basis, as a reported "chill" might be of any degree of severity with imperceptible transition into a completely healthy state. Furthermore, a certain transition in judgement standards cannot be excluded when observations extend over so long a period. Without doubt this constituted a possible sources of error in the elucidation of the principal question involved in this investigation, namely whether the man's physical condition deteriorated during the course of the observations as a result of the work. For avoidance of these sources of error, Table 10 summarises the means of the pulse-rates during all the 30 first and 30 last observation days including even the days designated as "not-normal". It is seen that the averages for the later time periods are lower in all five cases than at the beginning of the observations. For the subject J. S. the difference in pulse-rates between these periods is probable, for O. F. highly probable, and for the remaining

¹) Terminology: 0.05 > P > 0.01 probable
0.01 > P > 0.001 highly probable
0.001 > P significant

P indicates the probability of getting a value of the quotient of variances of at least the value actually found on the assumption of a common population.

TABLE 10. *Analysis of variance of the resting pulse-rates lying in bed, fasting. Means for the first 30 and last 30 observation days.*

Subject	Period	Average pulse rate	Quotient of variances	P
E.L.	19.3—4.5 1945	50.3	32.76	<0.001
	15.11—20.12 1945	45.3		
J.O.	17.10—23.11 1944	49.2	58.94	<0.001
	9.11—20.12 1945	45.7		
J.S.	14.11—20.12 1944	53.2	5.91	0.05—0.01
	11.10—17.11 1945	51.7		
B.N.	9.10—11.11 1944	53.4	152.5	<0.001
	14.11—20.12 1945	45.7		
O.F.	16.2—23.3 1945	51.7	8.59	0.01—0.001
	14.11—20.12 1945	48.9		

three significant. The systematic tendencies thus agree with those shown by the periods of normal-days.

The higher pulse-rates exhibited during the earlier part of the observation time may be ascribed to several causes. Partly they may depend upon the fact that the subjects were unfamiliar with the experimental procedure with resulting increase above the basal level from psychic causes. Partly, also, the cause may have been a training effect of the work which would thus show that an improved adaptation of the circulation had occurred. The first explanation is unlikely because, among other reasons, the man J. S., who was by no means the most stable psychically, showed the least statistically significant and the least absolute reduction of pulse-rate in spite of the fact that he entered the present experiments as a newly employed subject without any experience whatever of earlier experiments. On the other hand J. O. and B. N., who both showed a marked reduction, had previously served as experimental subjects involving morning pulse determinations for more than six months. It is further highly improbable that an effect of psychic origin

should operate so slowly as with the subjects where the gradual sinking of pulse-rate extends over the whole observation period.

As a consequence of this, the gradual reduction in the resting pulse-rate may be regarded as a training effect caused by the time-schedule work used during the investigations, the training effect being thus greater than the former occupations of the persons concerned. One of the subjects, B. N., had been engaged on rather light work as surveyor's assistant for about five months immediately preceding the commencement of these studies, so that the effects in his case find a natural explanation as training effects. The others, on the contrary, came direct from a long period of lumber work with some agricultural work in their own districts, so that the pulse-rate reductions in their cases signifies that lumber work had more training effect when time-scheduled than when carried on according to the men's own volition.

Training effects may be explained in two ways. Either lumber work in the home district of the person concerned is physically lighter, or the opposite may have been the case so that a certain degree of overtraining was present at the beginning of the investigation. Which of these two explanations is the correct one cannot be decided.

Concerning the principal question of these investigations the result may therefore be stated that in none of the experimental subjects could any functional deterioration be detected by this pulse-rate method.

"Not-normal" days: The days on which the pulse-rates were not reckoned in the foregoing account of normal-day periods have been arranged as far as permissible in the following groups for the purpose of closer analysis:

- 1) When the person concerned mentioned that he had a "chill".
- 2) When any considerable activity involving athletic movements or sport had been performed during the previous evening.
- 3) When spirits had been taken on the previous night.
- 4) When the subject had gone to bed later than mid-night on the previous evening.

For each of these groups the mean of differences of the resting pulse-rate from the basal level corresponding to the normal-day periods has been calculated. This was done in the following manner. For each day in the group the difference of the pulse-rate from the average pulse-rate of the concurrent normal-day period was determined. The standard error for the mean of these differences (\bar{d}) was then calculated according to the following formula:

$$E(\bar{d}) = \frac{1}{N} \sqrt{S \left[r_p \cdot \sigma_p^2 \cdot \left(1 + \frac{r_p + 1}{2 n_p} \right) \right]},$$

where r_p = number of "not-normal" days of subject concerned, during the corresponding normal day period.

$N = S(r_p)$.

σ_p = the standard deviation for the corresponding normal day period, and

n_p = number of normal days comprised in the normal day period.¹

¹) The formula is derived in the following manner. Assume that we have n_p "normal" values ($x_{p1}, x_{p2}, \dots, x_{pn_p}$) and r_p "not normal" values ($y_{p1}, y_{p2}, \dots, y_{pr_p}$), in the period p . The difference of the not normal days from the normal is measured by

$$\bar{d} = \frac{S_{p, q=1}^{r_p} (y_{pq} - \bar{x}_p)}{N}$$

where $\bar{x}_p = \frac{1}{n_p} S_q (x_{pq})$ and $N = S_p (r_p)$. Replace each term by

$(y_{pq} - m_p) - (\bar{x}_p - m_p)$,

where m_p is the real, unknown mean of the x_p 's. The standard deviation of the x_p 's from this mean is denoted by σ_p . If the hypothesis of a common distribution of the x_p 's and the y_p 's is true, these parameters will hold true even for the y_p 's, and we thus obtain

$$E^2(\bar{d}) = \frac{1}{N^2} \cdot S_p \left[r_p \left(\sigma_p^2 + \frac{\sigma_p^2}{n_p} \right) + \left(\frac{r_p}{2} \right) \frac{\sigma_p^2}{n_p} \right]$$

The results are set out in Table II. It will be seen that the number of days when the men complained of "chills" was rather large. This is explained to some extent by the fact that very light "chills" were included even when they did not cause any trouble to the man concerned. It is also probable, however, that lumber workers, accustomed to work out of doors all the year round under relatively unfavourable conditions, become hot and sweating during their work, so that in general a relatively high incidence of "chills" is to be reckoned with. Possibly this factor is also reflected in the work group under consideration, in spite of protective measures such as rest-huts and rain proof clothing. It should also be mentioned that

TABLE II. *Variations of the resting pulse-rate from the normal levels, during days of "chill", after evening athletics, consumption of spirits on the previous night, and after insufficient sleep.*

Group	Subject	Number of days in the group	Mean of differences of the pulse-rate \bar{d}	Standard error of the mean of differences ϵ	\bar{d}/ϵ
Chill	E.L.	42	+4.9	0.38	12.9
	J.O.	19	+4.2	0.48	8.8
	J.S.	51	+3.4	0.33	10.3
	B.N.	23	+3.8	0.49	7.8
	O.F.	49	+4.6	0.37	12.4
Evening athletics	all	21	+0.25	0.49	0.51
Consumption of spirits	all	24	+6.4	0.47	13.6
Insufficient sleep	all	28	+3.8	0.43	8.8

By a simple transformation we then arrive at the formula stated in the text above. In the calculations the σ_p 's are estimated from the corresponding empirical parameters for the normal values. The quotient \bar{d}/ϵ is to be regarded as approximately normal with regard to the number of observations in each group.

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"chills" were frequent even among the other personnel and may be ascribed to their unfamiliarity with the climate of the district. The "chills" recorded were all of the type uncomplicated infection of the upper respiratory tract, and only in isolated cases did they necessitate absence from work. It will be seen in table 11 that the pulse-rates on days when the man was suffering from a "chill" were significantly raised above the level corresponding to normal days.

In the group of resting pulse-rates after participation in sport or athletics on the previous evening, only such cases are taken into account where the activities were of an intensive nature such as football, middle-distance running, etc. Even the bodily exercise involved in these was not more strenuous than in the cases denoted by the term athletic exercise. Otherwise the sport in the cases referred to was limited to about half an hour almost every evening during the warmer seasons of the year. To obtain the appropriate total number of days here as in both the two following groups of "not normal" days, the means of differences have been calculated for all the experimental subjects. These are given in Table 11 where it is seen that evening athletics have no systematic influence on the resting pulse-rate next morning.

The group which deals with the resting pulse-rate after the drinking of spirits on the previous evening includes all the subjects except J. O. who was a teetotaller. It is apparent that in this group a significant increase of resting pulse-rate occurs. It should also be mentioned that on repeated occasions the taking of spirits on Saturday night was observed to leave an after effect of increased pulse-rate on Monday morning in spite of an intervening Sunday spent very quietly.

The last group includes pulse values after insufficient sleep during the night, the origins of the disturbed slumber being vague and sometimes indefinable, but often ascribed to mathematics lessons, card playing, visits to dance halls, cinemas etc. The results are thus not comparable with those found in standardised fatigue studies where interruption of sleep has been provoked (HENRY 1942, HOCHREIN and SCHLEICHER, 1944). The group has been included, however, as the cases comprised offer some examples of the influence of various activities which often encroach on the

night's rest in daily life and because, together with the other groups, they afford an opportunity of comparing the effects on the pulse and on the Schneider index. It is seen from Table 11 that in this group also there occurred a significant increase in the resting pulse-rate.

Resting arterial blood pressure

Earlier investigations

Earlier investigations on the resting blood pressure in relation to bodily work deal principally with the changes after effort. Thus FRIES (1908) found reduced values in Marathon runners both for the systolic and the diastolic pressures but the values previous to the races do not seem to be representative of resting conditions. HERXHEIMER (1933) describes corresponding reductions after middle-distance running which could still be observed 24 hours after the run. GUKELBERGER (1939) found that after ski-running 17 and 50 km. there was a considerable rise of the diastolic pressure of those runners who showed the best results whilst the others showed a reduction.

During investigations under basal conditions BROUHA and HEATH (1943) were unable to demonstrate any significant difference between students trained as oarsmen and average students. According to KNEHR, DILL and NEUFELD (1942) the blood pressure was unchanged during six month's training, but COGSWELL, HENDERSON and BERRYMAN (1946) found during a training period a trend downwards for the resting systolic and diastolic pressures in sitting position.

In fatigue states caused by loss of sleep HOCHREIN and SCHLEICHER (1944) found that the arterial resting blood pressure did not deviate from the level of that in the fully rested condition. They state, nevertheless, that labile values are often found after lack of sleep. Although it thus appears from the literature that there is no direct relation between the resting blood pressure level and the functional state, it was nevertheless considered justifiable in the present investigation to make such blood pressure measure-

ments, partly to follow it during long continued bodily exertion and partly as a factor in the general health control. Furthermore blood pressure is a factor in the Schneider index.

Methods

The measurements were made whilst the subjects were lying in bed immediately after the daily morning pulse counts. It was done by auscultation with a RIVA-ROCCI cuff equipped with an aneroid manometer which was calibrated several times during the observation period against a mercury manometer. The systolic pressure was read as that corresponding to the first sound heard during decompression, and the diastolic value as the lower limit of the range of strongest vascular sounds.

It was often noticed that the day's first measurement gave rather high values. This was specially true for J. S. whilst B. N. showed the least pronounced tendency in this respect. Every determination was therefore repeated until a basal level was reached. At least three and often more measurements were thus made.

Results

Normal days: The measurements have been collected in averages for period of 3 to 5 weeks coinciding with those for the resting pulse as already described, the "not normal" days being excluded here as before. The Tables 12 and 13 give the normal day averages for the systolic and diastolic pressures together with the standard errors of the means. The standard deviation for single determinations for the various periods varied between 2.3 and 7.4 for the systolic pressure and 3.1 and 7.2 for the diastolic.

The means are also represented graphically in Fig. 2 to 6 where it is seen that in no case has there been any pronounced tendency to alteration. The level from period to period fell within the limits which are to be regarded as normal. Analysis of variance (Table 14) shows that for the subjects E. L. and J. S. variations between the

TABLE 12. *Systolic blood pressure in recumbent position in the morning before food intake. The means and their standard errors for periods of normal days.*

Period No	Subject				
	E.L.	J.O.	J.S.	B.N.	O.F.
1				104.2±1.0	
2		109.0±0.6		106.9±0.9	
3		110.0±0.7	114.5±0.9	102.4±1.1	
4		107.8±0.8	114.3±0.8	102.7±0.6	
5		110.8±0.6	116.3±0.9	103.4±0.8	110.7±0.8
6	111.8±1.3	110.6±1.2	117.1±1.4	101.6±0.9	106.8±1.0
7	113.3±1.2	111.5±0.8	117.5±1.8	98.4±1.0	109.5±1.4
8	111.1±1.4	110.4±1.2	115.4±0.7	97.0±1.2	108.9±0.5
9	108.7±1.2	110.9±1.4		100.2±1.4	107.5±0.6
10	109.5±1.7	112.3±1.1	115.0±1.1	101.0±1.1	107.9±1.2
11	111.0±1.7	110.2±1.7	113.8±1.5	101.4±1.4	107.5±0.8
12	113.9±1.2	109.6±1.4	118.6±1.0	101.6±1.6	112.3±1.0
13	113.0±1.5	112.9±1.3	114.5±1.6	99.2±1.4	111.0±1.5
14	111.8±1.4	112.2±0.9		102.3±1.3	109.8±1.0

TABLE 13. *Diastolic blood pressure in recumbent position in the morning before food intake. The means and their standard errors for periods of normal days.*

Period No	Subject				
	E.L.	J.O.	J.S.	B.N.	O.F.
1				66.9±1.2	
2		69.4±0.7		68.2±0.7	
3		69.7±0.9	69.9±1.1	62.0±1.1	
4		65.9±1.1	69.2±0.8	62.7±0.8	
5		71.9±0.7	71.3±0.7	65.6±0.9	69.8±0.8
6	72.8±1.4	73.6±1.3	71.4±1.4	63.9±0.9	68.1±1.2
7	75.0±1.6	71.7±1.2	72.3±1.0	60.8±1.0	68.3±1.3
8	74.3±1.4	72.1±1.0	71.1±1.0	61.1±0.9	68.6±1.1
9	71.8±1.4	72.0±1.5		61.7±1.2	68.5±1.1
10	75.6±1.9	75.6±1.2	70.7±0.7	64.1±1.0	68.3±1.0
11	74.4±1.4	73.4±1.8	69.1±1.0	64.0±1.5	69.0±0.8
12	75.4±0.9	74.2±1.1	71.9±0.7	64.9±1.5	70.8±0.8
13	76.8±1.3	74.4±1.1	68.5±0.9	61.4±1.4	67.4±1.2
14	75.4±1.2	74.3±1.1		63.3±1.2	68.1±0.8

TABLE 14. *Analysis of variance of the arterial blood pressure (recumbent position in the morning before food intake) within and between periods of normal days.*

	Subject	Degrees of freedom		Quotient of variances	P
		1	2		
Systolic pressure	E.L.	8	143	1.47	0.2 — 0.05
	J.O.	12	226	2.25	0.01 — 0.001
	J.S.	9	203	1.93	0.2 — 0.05
	B.N.	13	270	5.15	< 0.001
	O.F.	9	172	3.20	0.01 — 0.001
Diastolic pressure	E.L.	8	143	1.27	> 0.2
	J.O.	12	226	7.17	< 0.001
	J.S.	9	203	1.77	0.2 — 0.05
	B.N.	13	270	4.20	< 0.001
	O.F.	9	172	0.95	> 0.2

periods might have been randomly distributed both for systolic and diastolic pressures. For J. O. it may be regarded as highly probable that the variations are due to systematic changes in the systolic pressure, whilst for the diastolic pressure the changes may be designated as significant. The subject B. N. showed significant differences between the periods for both pressures, whilst it is highly probable that the differences for O. F. represent a real change for the systolic but they are not significant for the diastolic pressure.

Although in certain cases there were thus systematic variations between the periods, these may not be related to the load of work because they partly range within normal limits and because there is no uniform trend during the course of the total observations.

"Not normal" days: Determinations here were made only for systolic pressures. The arrangements of groups and time periods were identical with those employed for the pulse-rate and the standard errors of the means of differences were determined in the same way.

TABLE 15. *Variations from the normal levels of the systolic blood pressure in the morning in recumbent position before food intake, during days of "chill", after evening athletics, consumption of spirits on the previous night, and after insufficient sleep.*

Group	Subject	Number of days in the group	Mean of differences of the systolic blood pressure \bar{d}	Standard error of the mean of differences ϵ	\bar{d}/ϵ
Chill	E.L.	42	+3.17	0.94	3.4
	J.O.	19	+1.76	1.09	1.6
	J.S.	51	+1.52	0.87	1.7
	B.N.	23	+1.54	1.07	1.4
	O.F.	49	+2.21	0.68	3.3
	all	184	+2.11	0.41	5.1
Evening athletics	all	21	+0.23	1.03	0.22
Consumption of spirits	all	24	+3.72	1.08	3.4
Insufficient sleep	all	28	+0.24	0.96	0.25

Results are given in Table 15. On days of "chills" there was a significant rise when the calculation was performed on all the subjects taken together. This was also true for the two subjects E. L. and O. F. taken separately whilst the deviations from the normal level of the remainder might only have been at random. In the groups after evening athletics and after insufficient sleep the levels are normal. The mornings after taking of spirits on the previous evening show a significant rise in resting systolic blood pressure.

Schneider Index

Earlier investigations

CRAMPTON (1905) worked out a physical test based on the pulse-rate and systolic blood pressure in recumbent and erect position. These scores are also included in the SCHNEIDER Index (SCHNEIDER 1920) which consists of a summation derived by allocating points for the pulse-rate when lying down and when standing, for the difference between these values, for the pulse reaction to standard work (stool stepping), and finally for the difference between systolic blood pressure in lying and standing positions. The statistical relations between the various factors entering into the index are discussed by SCHNEIDER and TRUESDELL (1922). The factors have further been discussed by MC CLOY (1931) who points out that the use of the bi-serial "r" method enables formulae to be derived on the basis of the same simple variables which constitutes the Schneider index but which are better correlated to the physical capacity.

Concerning the question of the value of the index SCHNEIDER states that it may be regarded as complementary only to the general clinical examination and that an index below 8 imperatively requires a more thorough investigation of the case.

The Schneider index has subsequently been the subject of a series of parallel investigations with other clinical observations. FEIL, PETTI and PARK (1943) found that it gave very little information on questions of cardiovascular reserve of patients with known cardiovascular diseases. TAYLOR and BROWN (1944) conclude, on the basis of a review of the literature that the Schneider index is only of value for the comparison of selected fitness groups and that it is inadequate when applied to individual cases. From a comparison with the maximal working capacity of 325 young men

they found that the index gave no certain evaluation of this. The inaccuracy of simple tests like the Schneider index has also been pointed out by DILL (1943).

SCOTT, BAZETT and MACKIE (1940) found that on the same subject there sometimes appeared pronounced variations in the different factors in the index between morning and evening experiments. Thus the former could give an index rising from day to day whilst the evening values could show an opposite course. They nevertheless state that, when the values for the same time of day are compared, the test may be capable to some extent of demonstrating changes in an individual's working capacity, but a comparison between different individuals gives uncertain results.

Methods

The tests were carried out according to SCHNEIDER's instructions (1920) by which pulse-rate and blood-pressure in a lying position were measured as previously described before the subject rose in the morning.

The evaluation of the various findings has been done according to the usual scale of point values. The resting pulse-rate in this scale is graded lowest for 50 beats per minute but lower values were frequently encountered in this investigation. The same applies to the standing pulse-rate where the lowest scale value corresponds to 60 beats per minute but values below this were often met with here. It would be desirable of course to define more closely the scale of points for the special purpose of this investigation. Our material nevertheless did not offer any basis for such a procedure so that it was necessary to apply the scales as they are at present, the given group limits being extended downwards.

Results

Normal days: Table 16 shows the means and their standard errors for the Schneider index during the above defined normal day periods of 3 to 5 weeks duration. The standard deviation for

TABLE 16. *Schneider index. The means and their standard errors for periods of normal days.*

Period No	Subject				
	E.L.	J.O.	J.S.	B.N.	O.F.
1				16.2±0.3	
2		15.8±0.2		16.1±0.2	
3		15.7±0.2	14.7±0.3	16.5±0.2	
4		14.9±0.2	14.6±0.3	16.3±0.2	
5		15.4±0.2	14.3±0.3	15.5±0.3	14.3±0.3
6	13.3±0.4	15.6±0.2	14.3±0.5	14.8±0.2	13.4±0.3
7	13.8±0.4	15.1±0.3	14.9±0.5	15.7±0.3	13.2±0.4
8	14.1±0.3	15.6±0.3	13.7±0.3	15.8±0.4	14.8±0.4
9	15.1±0.3	15.2±0.5		15.5±0.5	15.5±0.3
10	14.8±0.5	16.6±0.3	14.8±0.3	16.1±0.3	15.1±0.4
11	14.5±0.4	16.4±0.3	14.2±0.6	15.7±0.4	15.4±0.3
12	14.2±0.4	15.9±0.3	14.0±0.4	15.8±0.4	15.7±0.3
13	14.3±0.3	15.9±0.3		16.0±0.3	15.7±0.4
14	14.9±0.4	16.2±0.3	14.8±0.4	16.3±0.2	15.7±0.2

TABLE 17. *Analysis of variance of the Schneider index within and between periods of normal days.*

Subject	Degrees of freedom		Quotient of variances	P
	1	2		
E.L.	8	146	2.95	0.01—0.001
J.O.	12	226	4.02	<0.001
J.S.	9	198	1.08	>0.2
B.N.	13	266	2.46	0.01—0.001
O.F.	9	169	6.89	<0.001

single determinations in the separate periods varied between 0.7 and 2.2. The means are also given graphically in Fig. 2 to 6. It is seen, that they varied rather slightly during the course of the observation period. To decide whether there was any systematic variation

TABLE 18. Means of the Schneider index during the first 30 and last 30 observation days, analysed by Student's *t*-test.

Subject	Period	Means of the Schneider index	<i>t</i>	Degrees of freedom	<i>p</i>
E.L.	19.3—4.5 1945	13.03	3.916	58	<0.001
	15.11—20.12 1945	14.73			
J.O.	17.10—28.11 1944	15.60	2.042	58	0.05—0.02
	8.11—20.12 1945	16.13			
J.S.	22.11 1944—16.1 1945	14.63	0	58	1
	10.10—17.11 1945	14.63			
B.N.	3.10—7.11 1944	16.07	0.376	58	0.7
	14.11—20.12 1945	16.17			
O.F.	16.2—23.3 1945	14.30	3.502	58	<0.001
	14.11—20.12 1945	15.57			

between the separate periods analysis of variance was applied as before (Table 17). It is apparent that for J. O. and O. F. there was a significant variation and for E. L. and B. N. a highly probable variation between the groups, whilst for J. S. the variation was only a random distribution. Even where a systematic variation could be demonstrated it follows from the means that no pronounced long term tendency is concerned.

For the same reasons which operated where the resting pulse-rate was concerned, Table 18 has been constructed giving the average of the Schneider indices during all the 30 first and the 30 last days of the total observation period, irrespective of whether these included any "not-normal" days. The differences between these periods have been analysed according to Student's *t*-test. It is thus gathered that the Schneider index for E. L. and O. F. were significantly higher for the later period, while for the others the level was unchanged.

"Not-normal" days: Similarly "not-normal" days were arranged

TABLE 19. *Variations of the Schneider index from the normal levels, during days of chill, after evening athletics, consumption of spirits on the previous night, and after insufficient sleep.*

Group	Subject	Number of days in the group	Mean of differences \bar{d}	Standard error of the mean of differences ϵ	\bar{d}/ϵ
Chill	E.L.	42	-0.31	0.26	1.19
	J.O.	19	-0.51	0.25	2.04
	J.S.	51	-0.43	0.28	1.54
	B.N.	22	-0.20	0.24	0.83
	O.F.	48	-0.17	0.22	0.77
	all	182	-0.31	0.12	2.58
Evening athletics	all	21	-0.01	0.29	0.03
Consumption of spirits	all	22	-1.48	0.35	4.23
Insufficient sleep	all	28	-0.88	0.27	3.26

in separate groups. The fact that some of these groups comprise one to two days less than the corresponding pulse groups is due to the absence of measurements for these days. These omissions are insignificant in relation to the total number of days within the groups.

Table 19 gives the results here. It appears that the Schneider index during days of "chills" does not deviate significantly from the normal level for any of the subjects. Although the means of differences throughout are negative their magnitudes are only from 0.8 to 2.0 times the standard error. Nor were any systematic differences from the normal level noticed, when the calculation was made on all the subjects together.

Similar results were gained during mornings after evening athletics. The taking of spirits on the previous evening and also "insufficient sleep", on the contrary, gave negative means of differences which were for the respective group concerned 4.2 and 3.3 times the standard error.

The Functional Capacity of the Oxygen Transport Apparatus during Work

The clinical investigations upon pulse and blood pressure described above can of course only give indirect information concerning the working efficiency of the body, whilst a direct measurement could be done only during work. Certainly, the reaction to effort is a component in the SCHNEIDER index but the results of this test are not unambiguous. For these reasons it was necessary to apply a test which would record the bodily reaction during actual work and when selecting the method the aim was to find one which would indicate the degree of adaptation of the oxygen transport apparatus.

The choice was necessarily influenced by certain practical conditions. It was desirable that the test should not interfere in any way with the ordinary working time of the subject, nor should it be so fatiguing as to lower his output in the forest later in the day. The test finally selected was a submaximal series of loads on the bicycle ergometer, the intensity of work being expressed in terms of the oxygen consumption and the latter being correlated with the pulse-rate and the ventilation coefficient during work. Before proceeding to the detailed description of this, however, the range of the physical capacity tests employed by previous authors, and their accuracy and advantages and disadvantages may briefly be reviewed.

Discussion of various criteria of the working capacity

The tests of physical capacity given in earlier literature are of two kinds, namely, maximal and submaximal tests.

Maximal tests: For these the experimental subject performs work involving subjectively maximal effort as regards speed, load, duration or total amount of work. Points are allotted either for the external work done or for the physiological response in several aspects. In ordinary life as well as in industrial work the range of bodily effort very seldom involves so great a stress upon the body as to lead to exhaustion. Maximal tests are nevertheless justifiable because they give the limits of the physical capacity and thus enable the reserve power to be determined. It must be expected that a man who can perform a given amount of work with a large measure of reserve power will be less affected by the effort, less fatigued and suffer less bodily depletion.

It is when peak-effort is involved that the theoretical advantages of the method and its practical weaknesses become apparent. The most suitable basis for comparative tests is undoubtedly the maximal external effort, as this elicits fundamental reactions of various physiological factors. The definition of peak-effort is itself, however, a source of uncertainty where routine quantitative functional tests are concerned. This cannot of course be determined by physical factors alone; the subjective feelings and stress which the person concerned will tolerate must be taken into account. Such psychic factors undoubtedly manifest themselves in tests upon highly co-operative and personally interested subjects and are of still greater importance in experiments on people in general as in certain cases malingering has naturally to be reckoned with. Maximal tests are thus of importance as fundamental laboratory tests, whilst standard clinical experiments are directed to tests which do not involve the measurement of maximum capacity, because of the difficulty of defining this exactly. It is nevertheless desirable that sub-maximal tests should be of such a kind as to permit extrapolation to yield the maximum physical limit.

As far as the various point scores for maximal tests are concerned, JOKL and CLUVER (1941) consider the external work-maximum to be satisfactory (speed, load or duration). TAYLOR (1944) found from treadmill work which was stepped up to exhaustion that the working time until exhaustion which he calls the "performance index" was more reliable ($r=0.95$) as a measure

of the physical capacity than any physiological measurement. This agrees in main with the results of JOHNSON, BROUHA and DARLING (1942).

FOLTZ, IVY, BARBORKA and SCHIFFRIN (1942, 1943) recorded the maximum external work performed in two periods separated by an interval usually of ten minutes. As point scores they used the recovery percentage derived from the ratio $100 \times \text{work II} / \text{work I}$. Their method has been criticised by TAYLOR (1945), who found that its consequences lead to contradictions in the light of some of the authors' own experimental findings.

The maximum external work during athletics has further been used as a score in some of the literature cited by TAYLOR (U. S. WAR DEPARTMENT 1942, LARSON 1942, HUGHES 1942, KISTLER 1944, HEADQUARTERS, U. S. ARMY AIR FORCES 1944). During an examination of county jail inmates KARPOVICH and PESTRECOV (1941) found that the duration of work at a constant load on the bicycle ergometer showed a considerable increase during the course of a training period. But when investigating people in general one must reckon with the fact that work of long duration may be cut short by psychological factors, as suggested by SIMONSON and SIRKINA (1933).

Physiological scores during maximum work may be derived either from the energy metabolism, the oxygen consumption, the degree of anaerobic energy liberation as well as from different circulatory and respiratory factors.

Certainly, the oxygen transport capacity represents an essential condition for the performance of external work and athletes highly trained for long performance have the largest oxygen consumption rate. HILL, LONG and LUPTON (1924) regard the oxygen consumption during maximum work as a good measure of the limitations of the circulatory-respiratory system. In the treadmill experiments of TAYLOR (1944) cited above, however, there was only a moderately positive correlation between the maximum running time, and the maximum oxygen intake although this correlation was higher than for the majority of other factors. DILL (1942) showed that the maximum capacity for oxygen intake decreases with increasing

age, and he ascribed this to the diminished capacity for acceleration of the heart rate.

On *a priori* grounds it can hardly be expected that the anaerobic energy liberation at maximum working rate would be an expression of physical capacity but rather of the resolution of the subject fully to utilise his bodily resources. But ROBINSON and HARMON (1941) and KNEHR, DILL and NEUFELD (1942) found that the ability to accumulate lactic acid during anaerobic work increased with training. According to TAYLOR (1944), however, the blood lactate at maximal run shows no significant correlation with the time-run involved. JOHNSON and BROUHA (1942) found that subjects who could tolerate a certain speed for five minutes on the treadmill had a blood lactate content of 73 to 160 mg %, when they had to stop because of fatigue. In this group the lactate concentration was, however, not correlated with the working capacity. ROBINSON (1938) found that blood lactates after maximum work stand in a certain relation to age in that the highest values occur in the 30 to 40 years age group and that the capacity to accumulate lactic acid decreases with increasing age.

Considering the circulation, the heart's minute volume is undoubtedly the most suitable measure of maximum capacity, but the inherent difficulties of such absolute measurements prevent its routine use. Accordingly a relative index, the LILJESTRAND—ZANDER factor (1928), may be employed. This is derived very readily from pulse-rate and blood pressure determinations and gives an index of the trend of cardiac output.

According to BORGARD (1937) the pulse corresponding to maximum work seldom rises above 180 beats per minute in physically well trained persons. With untrained subjects and cardiac patients he observed, however, a higher maximum-work pulse-rate. ROBINSON, DILL, HARMON, HALL and WILSON (1941) found a negative correlation between the ability to consume oxygen and the maximal heart rate of fiftyeight subjects, aged 16 to 24 years. But TAYLOR (1944) states that the pulse-rate is not significantly correlated with the maximum running time on the treadmill.

JOHNSON and BROUHA (1942) in the experiments cited above found that the group of subjects who could not stand up to 5 mi-

minutes work in the treadmill, finished the work with a pulse-rate of 190 to 200, although pulse-rate differences within the group did not enable any further gradation of the physical capacity to be made. The pulse recovery after maximum work is discussed by KNEHR, DILL and NEUFELD (1942) who found that it could not be used as a functional test. SIMONSON, ENZER, BAER and BRAUN (1942) showed that the time of pulse-rate retardation after a given amount of work repeated once each week could diminish without change in the work capacity as measured by the work time.

The oxygen pulse (oxygen consumption per pulse beat per kg body weight) is stated by TAYLOR (1944) to have the same correlation with the maximum running time in the treadmill as has the maximum oxygen consumption.

Lung ventilation and respiratory rate in maximum work are not significantly correlated with working capacity, according to TAYLOR (1944). This is readily understood because these factors, like the maximum pulse-rate, are not direct expressions of the capacity of the body, but a reaction to the work in question. They are thus rather an indication of the extent to which the subject has exerted himself. As HOHWÜ CHRISTENSEN (1944) states, a respiration rate of 30 to 40 shows that the subject has almost reached the limit of his working capacity. But even in this respect the point scores are uncertain because average levels are lacking for different sexes, age-groups, constitutional types, occupations etc.

Submaximal tests: These consist of measurements of the relation between the heaviness of the work and the physiological response. The heaviness of work can be stated in terms of external effort (intensity, duration, total work, speed etc.), or in terms of oxygen consumption or energy metabolism. The latter measurements are to be preferred if the capacity of the oxygen transport apparatus is to be measured without ambiguity. Moreover these give results independent of the efficiency with which the work is done. Efficiency may vary considerably in different persons and even in the same person it may show changes in repeated tests because of training effects of the special test work, and this without any concomitant change in the adaptation of the oxygen transport system (BERGGREN and HOHWÜ CHRISTENSEN 1943). It must be

admitted, of course, that working efficiency is an integral part of the practical utilization of physical capacity in daily life. But the co-variation between efficiency during test work and the occupations of daily life is difficult to estimate although the difficulties become less the more closely the test work resembles the ordinary daily occupation (walking on treadmill, ascending stairs etc.).

Submaximal tests may be done either with one standard load or with an increasing series of loads. The latter is to be preferred because the results may then be plotted as a curve instead of giving a single point merely. Besides the gain in precision, the necessity of making exact determinations of the external work is avoided, approximate loads being sufficient for which the effort is exactly determined with the aid of physiological data.

As TAYLOR (1945) states, all submaximal tests must have their reliability controlled by correlation with reasonable criteria of the working capacity, and in addition their sensitivity must be determined. TAYLOR and BROZEK (1944) point out the desirability of standardising the various tests so that data from different laboratories may be compared. The most usual object of comparison is the maximum external work, but other methods have been tried. Thus HENRY (1942) uses the effect of a simple cold.

Among circulation factors which may conceivably be used as scores in submaximal tests the minute volume of the heart may be at first mentioned. According to a large number of investigations (NEWBURGH and MEANS 1915, BOOTHBY 1915, LINDHARD 1915, COLLETT and LILJESTRAND 1924, ROCK, VANCAULAERT, DILL, FÖLLING and HURXTHAL 1928, HOHWÜ CHRISTENSEN 1931) the minute volume stands in linear relation to the oxygen consumption. COLLETT and LILJESTRAND found in this connection, that work corresponding to a given oxygen consumption was done with a somewhat lower minute volume in better trained subjects. This has been confirmed by HOHWÜ CHRISTENSEN, who states, however, that when the work is done using the same muscles and in the same posture the somewhat higher minute volume for given oxygen intake can be found only in completely untrained subjects. MÜLLER (1942-43) considers on the basis of pulse observations, that work involving the use of trained muscle groups is done with a lower minute volume

for given oxygen used, than with untrained muscles, and ascribes this to improved vascularisation of the muscles as demonstrated by PETRÉN and SJÖSTRAND (1934). MÜLLER's assumption does not find experimental support in the experiments by HOHWÜ CHRISTENSEN and others. The different pulse reactions during work with varying muscle groups can well be explained as due to different hydrostatic conditions for the return of venous blood to the heart. In conclusion it thus appears that the minute volume not only involves difficult methods but is also inapplicable on other grounds as a point score factor in sub-maximal tests.

BOOTHBY (1915) and KROGH and LINDHARD (1917) showed that the heart rate is a linear function of oxygen intake while BOCK, VANCAULAERT, DILL, FÖLLING and HURXTHAL (1928) found that this did not quite hold during higher levels of work. These authors, as well as HENDERSON, HAGGARD and DOLLEY (1927) showed a lower working pulse-rate at well-trained persons. According to HOHWÜ CHRISTENSEN (1931) there is a gradual reduction in pulse-rate for given load of work during the training process, the degree of reduction depending upon the heaviness of the training work. A decrease in working pulse-rate during training has also been shown by ERICKSON, SIMONSON, TAYLOR, ALEXANDER and KEYS (1946).

Thus in any one individual the pulse-rate during work seems to be a good index of functional condition. For comparison between different individuals TAYLOR (1944) states that it, together with the blood lactate content, is the most reliable score for submaximal tests as the correlation when walking in a treadmill was $r = -0.56$ to the maximum running time.

WAHLUND (1945) used the working pulse-rate in a well standardised test adapted for clinical use. His test consisted of an uninterrupted series of performances upon a modified KROGH bicycle ergometer. The initial load was 300 or 600 kg.m./min. and was increased every seven minutes by 300 kg.m. up to 1200 kg.m./min., if the subject could reach this. In addition to the pulse-rate the ventilation coefficient was determined in a large series and a good co-variation was obtained between the changes of these factors.

In common with all functional tests the pulse-rate during work

admittedly gives information only on the actual functional level so that its value is limited when differential diagnosis and prognosis of pathological conditions of the respiratory and circulatory systems are concerned. Thus ALBERS (1942) found during ergometer tests on cardiac patients according to the method of KNIPPING (1929) that the type of heart lesion had no essential effect on the rise of pulse-rate. ESKILDSSEN (1945) carried out a comparison between normal subjects and cardiac and asthmatical patients using KROGH's bicycle ergometer. He came to rather pessimistic conclusions as to the value of the various physiological responses, including the heart rate, for diagnostic or prognostic purposes or for functional evaluation. Truly deviating values were in general obtained only in such advanced stages that they could be clearly recognised by usual clinical methods because the limits of the normal values were so wide.

Pulse recording in relation to work has also been used in other connections than the above. MÜLLER (1943) takes the pulse quo-

tient, $\frac{\text{pulse-rate}}{25 \cdot 10^{\log O_2}}$ as a measure of the stress which a given work

calls for. The interpretation which he gives to his quotient does not seem to be adequately supported by experiments.

KIBLER and BRODY (1943) give the average oxygen pulse as 0.06 and state that the residual variation about this level can be applied as an index of the capacity for muscular work. TAYLOR (1944) finds, however, that it has an insignificant correlation with the maximum work capacity when it is calculated from sub-maximal data. SCHNEIDER and CRAMPTON (1940) allot a certain value to the oxygen pulse for the estimation of the differences in stroke volume between trained and untrained subjects.

LEHMANN and MICHAELIS (1941) use the working pulse-rate in a test which, in its construction, differs from other common types. The work is done on a bicycle ergometer with increasing output, the starting output being adapted according to the calf circumference. The work is continuous until the product of pulse-rate per minute and systolic blood pressure in mm. Hg. reaches 10,000 and the score is determined by the time necessary for this.

Recording of the pulse-rate after graduated work enters into

many fitness tests, for example those of SCHNEIDER (1920), ABRAHAMS (1939), JOHNSON and BROUHA (1942) and GALLAGHER and BROUHA (1943). The latter found during a comparison of selected fitness groups, that the working capacity was inversely proportional to the pulse-rate and blood lactate content early in recovery. A test based upon this has therefore been adapted for field use, for example for comparison between men and women (METHENY, BROUHA, JOHNSON and FORBES 1942), for investigation of the effect of the masculine component (SELTZER and BROUHA 1943), and the effect of training (BROUHA 1943) and vitamin deficiency (WALD, BROUHA and JOHNSON 1942, KEYS, HENSCHER, TAYLOR, MICKELSEN and BROZEK 1945). It has also been employed on adolescent boys (GALLAGHER 1944) and girls (GALLAGHER and BROUHA 1943) and on young women (HARDY, CLARKE and BROUHA 1943). COGSWELL, HENDERSON and BERRYMAN (1946) found by examinations one to three times a week during a period of twelve weeks that the pulse-rates at one, two and three minutes after a submaximal step test showed a decrease with training, whereas maximal tests on the treadmill and the bicycle ergometer failed to produce a similar response of the post-exercise pulse. The resting pulse-rate in a sitting position also remained unchanged. Men whose resting pulse-rates were higher than the general average, or temporarily higher than their own average, tended to have higher than average post-exercise pulse-rates.

According to TAYLOR (1945) the JOHNSON—BROUHA test is only of value for the estimation of wide group differences and gives "very peculiar distributions of scores in representative populations". Tests in which the time for pulse retardation after work is involved (KELLY 1941) are regarded by TAYLOR as resting upon insufficient evidence.

MOREHOUSE and TUTTLE (1942) studied the pulse-rate after stool-stepping at different rates and found that the reliability of the results as tests of physical condition became acceptable only when the rate reached 40 to 50 steps per minute. At lower work-intensities the post-exercise pulse-rate was inversely related to the resting pulse-rate, the correlation changing only when the work became vigorous. This is confirmed by PHILLIPS, RIDDER and

YEAKE (1943). The influence of the bench-height on the pulse reaction to stool-stepping has been investigated by ELBEL and GREEN (1946).

It is well known that the respiration rate increases with increasing load of work, and that well trained men may be recognised by their good respiratory economy shown by a relatively low respiration rate both when working and resting. SIMONSON (1926—1927) nevertheless found that the correlation between respiration rate and load of work was rather loose, and TAYLOR (1944) also considers that with standard work there is no significant correlation with the maximum work capacity.

A more suitable score for the respiratory factor lies in the relation between ventilation and oxygen consumption. According to COLLETT and LILJESTRAND (1924) and HOHWÜ CHRISTENSEN (1931—32) this relation is at first linear when increasing loads are considered. When the load approaches the maximum for the individual and for the muscle group in question the linear relationship ceases, the ventilation increasing more rapidly than the oxygen consumption. This may otherwise be expressed by the statement that the lung ventilation per litre of oxygen intake (the ventilation coefficient) is constant for moderate loads, whilst for heavier loads the ratio is no longer constant but increases continuously.

It has also been shown that the ventilation coefficients for a given load of work are lower for well trained subjects than for others (COLLETT and LILJESTRAND, 1924; SIMONSON, 1926—27; MISSIURO, NIEMIECKO, PERLBERG and PAWLAK, 1939; SCHNEIDER and CRAMPTON, 1940; EBELING and LINXWEILER, 1940; BØJE, 1944). BARMAN, CONSOLAZIO and MOREIRA (1942) find a good correlation between the efficiency of ventilation during recovery after exercise and the general physical fitness for hard muscular work. WAHLUND (1945) uses the ventilation coefficient during work in his bicycle ergometer tests. At a moderate load of work the absolute values of the ventilation coefficients seem to be characterised by quite large individual variations which are probably produced by factors other than the physical capacity. In HOHWÜ CHRISTENSEN's results (1931—32) the range was 13.8 to 20 litres ventilation per litre of oxygen consumed (0° C, 760 mm, dry).

Higher ventilation values were reached for arm work than for leg work in the same subject for the same energy output. In ESKILDSEN'S (1945) investigations, previously cited, cardiac patients showed pronounced hyperventilation for heavy work, whilst asthmatical subjects had relatively low ventilation in spite of marked dyspnoea.

The composition of the alveolar air during work has been studied by ARBORELIUS and LILJESTRAND (1923), HOHWÜ CHRISTENSEN (1931—32) and by NIELSEN and ASMUSSEN (1944). It appears from their results that the alveolar air has no clear applicability as a score in physical capacity tests, and, moreover, the estimation is attended by uncertainties in subjects unfamiliar with the procedure.

The degree of anaerobic energy liberation during standard work has been employed in several tests, the oxygen debt being measured, (NYLIN 1933) or the blood lactates estimated. According to TAYLOR (1944) the latter, in association with the working pulse-rate, is the most reliable submaximal test. CRESCITELLI and TAYLOR (1944) found significant differences in average blood and urine lactate between selected fitness groups. In this connection it may be mentioned that SIMONSON and RIESSER (1926—27) and LIEBENOW (1928) found a quicker oxidative restitution after physical training.

Other changes associated with work may also be considered as possible scores for physical capacity measurements. Among these the blood alkali reserve may be mentioned. This diminishes during work, the extent of reduction during standard work being inversely related to the training condition (PARADE and OTTO, 1939).

The respiratory quotient during work is also related to the physical capacity in that the higher values found with increasing load of work (SMITH, 1922; HILL, LONG and LUPTON, 1924; BOCK, VANCAULAERT, DILL, FÖLLING and HURXTHAL, 1928; TALBOTT, FÖLLING, HENDERSON, DILL, EDWARDS and BERGGREN, 1928), are apparent at a less load with subjects not in physical training, and also when weaker muscle groups are used (HOHWÜ CHRISTENSEN and HANSEN, 1939). The authors clarify this in the following way: the rise in RQ occurs because the oxygen consumption approaches the limits of capacity for the individual and muscle

group concerned, with consequent transition towards a relatively greater carbohydrate combustion. This latter factor is associated with oxygen saving even because of the improved efficiency, an effect previously demonstrated by KROGH and LINDHARD (1920). Determination of the real RQ as carried out by HOHWÜ CHRISTENSEN (1931—32) for example, involves a method requiring great care and is therefore difficult to adapt as a standard test.

Among reactions which are dependent on the physical capacity, work proteinuria may finally be mentioned. This was demonstrated by FRIES (1907) after the running of various distances, and this author also found that it was most pronounced after shorter and therefore more intensive runs, down to 400 metres. Experiments of HOHWÜ CHRISTENSEN (personal communication) show that proteinuria first appears when the individual's maximum capacity is approached. It is thus possible that it could be applied as a physical test.

Summary of literature

The literature on the subject of functional tests is a particularly abundant flora and the foregoing survey only delineates the principal growth in this field of frequently changing experimental data. Certain of the experimental results which are at variance with one another should be capable of explanation as arising from differences in the experimental conditions. Maximal tests seem to have their value in determining the applicability and sensitivity of various physiological scores. As a general rule it follows that they should be carried out under strictly standardised conditions on workers familiar with the method. It is possible that the relatively low correlation which has been found in some cases between the different physiological reactions and the maximal external effort arises from the fact that the latter was not always truly maximal for the body.

For routine use it appears that submaximal tests are most suitable, and it is desirable that these should comprise an increasing series of loads in order that a continuous curve of the relationships may be constructed. It is self-evident that the statistical probability increases, the larger the range of the series of loads and the more

points on the curve. A large range is also of value for the reason that a test with moderate loads may place an individual in one fitness group, whilst heavier loads place him in another group. This follows because, for example, a pulse-rate test may yield low values with moderate work loads and a sudden increase of values when the load is increased. Thus this can occur with the working pulse-rate in a person with good physical training but moderate physical resources. It is thus imperatively necessary to state exactly what is meant by physical capacity, i. e. whether this refers to the degree of adaptation to more moderate loads corresponding to the demands of daily life, or whether the maximal capacity is meant.

From the literature it also appears that rather large individual variations occur when different factors are measured, and these cannot be explained by variations in the functional capacity alone. Moreover, as TAYLOR (1945) pointed out, the degree of co-variation between different qualities is not sufficiently high to enable any further degree of significance to be reached by any statistical combination index. It thus seems out of the question to seek any single reliable test at present. As a consequence of the lack of exactness of the individual test it is still necessary to employ more laborious procedures, determining function on the broadest experimental base possible and to supplement this with further indirect clinical observations and case-history data (cf. ESKILDSEN, 1945).

The uncertainty which attends the various work tests has its origin principally in the attempt to compare different persons and to relate them to a normal level. But if the attempt is to follow the developments in the same person as was done in these investigations, the accuracy is undoubtedly much greater.

Methods

During the first months of the observations experiments were done with one load only. In January 1945 the revised method was first applied so that the results given refer to the period from January to December, 1945.

Investigations were made on each subject approximately once a

week. The interval was, however, occasionally somewhat longer due to statutory public holidays, other morning tests etc. The days on which the tests were made were arranged so that these were fairly uniformly distributed on the different week days. The experiments were done on fasting subjects between six and seven o'clock in the morning. The journey to the laboratory, about 700 metres downhill, was made in quiet tempo on a bicycle, and on arrival the subject rested for about fifteen minutes.

The test work was done on the bicycle ergometer with band brake and gear ratio 3:1. Loading was done with a spring dynamometer connected to one end of the brake band. The other end towards which the wheel rotated was connected to a second spring dynamometer so that the effective load could be computed and continuously regulated during the experiment by observations on the difference in deflection of the two dynamometers. The arrangement did not permit the same possibility of exact adjustment to absolute work levels as is possible with KROGH's bicycle ergometer (1913), but nevertheless gave a constant work intensity in the separate experiments which was sufficient for the requirements of the investigation.

The series of loads was 1.0, 1.5, 2.0 and 2.5 kp for four of the men, whilst for the eldest, E. L., it was 0.5, 1.0, 1.5 and 2.0 kp. In single cases the series was extended upwards towards the limits of the man's capacity, the increase from one experiment to the next being 0.5 kp. The working speed was controlled by an electric cyclo-meter set to a pedalling rate of 117 per minute by means of a metronome. This corresponded to 176 revs/min. of the wheel. The men nevertheless showed a certain trend towards a somewhat lower tempo than that of the metronome. This was unimportant, however, because the working intensity was given in the oxygen consumption. Between each load there was a pause of about five minutes.

The carotid pulse-rate was estimated by counting for one half minute every minute after the commencement of the experiment. After five minutes the collection of expired air was begun, usually in a gasometer of 180 litres capacity but in isolated cases in a Douglas bag. Connections between the respiratory valves and the gaso-

meter or Douglas bag were made by means of tubes of 30 mm internal diameter and a three-way tap of corresponding diameter. The respiratory rate was counted after 5 minutes of working time.

Experimental Control of Method

In the present investigation it was desirable to reduce the time involved in an experiment, not only in the tests referred to but also in the determination of the intensity of energy metabolism during various forms of work in the forest. According to experiments of HOHWÜ CHRISTENSEN and HANSEN (1939) it appears that a work time of at least 10 to 15 minutes is necessary to yield a real respiratory quotient even for well trained subjects familiar with the procedure. Adequate oxygen consumption in experimental tests involving moderate work intensities on the contrary, occurs within a few minutes. This was also shown by BOCK, VANCAULAERT, DILL, FÖLLING and HURXTHAL (1928) and KNEHR, DILL and NEUFELD (1942).

As a study of the real RQ was not aimed at here, a time period for readings sufficient to ensure an adequate oxygen uptake was considered satisfactory. In such cases where the intensity of energy metabolism was calculated, an approximation was made by which a caloric coefficient for oxygen of 4.85 was taken throughout. The error which could thus arise (up to somewhat more than 2 lit. oxygen/minute) was certainly less than $\pm 2\%$ and can therefore be regarded as insignificant.

In order to determine how soon after the commencement of the work the oxygen uptake and pulse-rate level could be regarded as adequate, control experiments were done partly with lumber work and partly with the bicycle ergometer. In these the collection of expired air was done either immediately during a sequence of two minute periods or at periods of 5, 10 and 15 minutes after the beginning of the work. Fig. 7 shows such an experiment from lumber work. It will be seen that after a period of 1 to 3 minutes the oxygen consumption has already risen to 2.5 l/min., which level is maintained practically unaltered. Table 20 gives the results of expe-

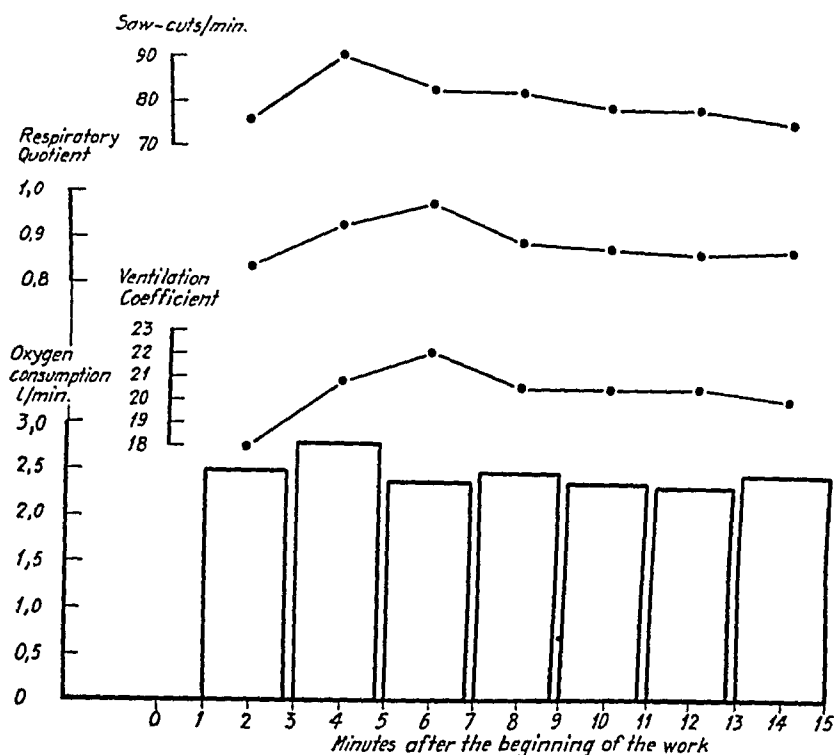


Fig. 7. Tree-felling. Collection of expired air at different times after the beginning of work. Subject B.N.

periments on the bicycle ergometer with work loads corresponding to, or somewhat higher than the highest used in the functional tests. From a comparison of the oxygen consumption in the first and third tests it appears that this remained unaltered for three of the men, whilst J. O. and O. F. showed a rise of 3 %. For J. O., who seldom succeeded in maintaining a constant rate of revolutions, this increase can be associated with the increased speed of work. For O. F. the explanation can either be inadequate oxygen consumption in the earlier tests or decreasing efficiency. Which explanation is correct cannot be decided from these experiments. Any larger excess anaerobic energy liberation is excluded by the rather slight variations of the respiratory quotient. In any case the deviations are so small as to be practically insignificant.

It was found to be important to perform the morning experi-

TABLE 20. *Work on the bicycle ergometer. Collecting of the expired air and counting of the pulse-rate at different times after the beginning of the work.*

Sub- ject	Sample after min.	Revs/ l/min.	O ₂ l/min.	Ventil- ation l/min.	Ventil- ation coef- ficient	RQ	Res-pira- tion frequency	Pulse rate
E.L.	5	170	1.95	39.2	20.1	0.88	27	115
	10	171	1.85	40.6	22.0	0.92	28	119
	15	171	1.96	41.8	21.4	0.88	30	119
J.O.	5	178	2.09	32.8	15.7	0.84	14	120
	11	183	2.14	34.9	16.3	0.85	15	124
	13	184	2.16	35.3	16.3	0.88	16	126
J.S.	5	176	2.07	43.4	21.0	0.88	29	119
	10	176	2.12	43.0	20.3	0.86	30	121
	15	176	2.07	42.8	20.7	0.88	29	122
B.N.	5	176	2.01	37.5	18.7	0.85	21	116
	10	175	2.01	37.9	18.9	0.86	19	116
	15	175	2.02	38.2	18.9	0.86	22	120
O.F.	5	178	2.24	41.5	18.5	0.90	21	142
	10	176	2.21	41.7	18.8	0.91	20	144
	15	178	2.31	42.5	18.4	0.89	21	147

ments with an increasing series of loads. In control experiments using a decreasing series of loads, adequate oxygen consumption at the highest loads did not occur with several of the men. This was particularly marked in the first experiment of the day, because with the same load later in the day five minutes working time was sufficient in spite of a previous resting interval of more than half an hour.

Comparison of the pulse-rates in the first and last tests as given in Table 20 shows that an increase of 3 to 6 beats per minute has occurred. Because several control experiments show that the initial and principal pulse acceleration occurs during the first 1 to 2 minutes of work, the 5 minute pulse determinations could be con-

TABLE 21. *The deviation of the pulse-rate, six minutes after the beginning of work, from the mean pulse-rate during the respiratory experiments with the bicycle ergometer.*

Subject	Load Kp.	Number of obser- vations	Mean of differences	Standard deviation of the mean of differences
E.L.	0.5	32	- 0.1	1.1
	1	30	- 0.2	1.1
	1.5	31	- 0.3	0.8
	2	31	- 0.4	1.0
J.O.	1	32	+ 0.1	1.2
	1.5	31	- 0.5	1.1
	2	32	- 0.3	1.0
	2.5	30	- 0.4	1.0
J.S.	1	41	- 0.4	0.9
	1.5	41	- 0.5	0.8
	2	39	- 0.5	0.8
	2.5	32	- 0.4	0.8
B.N.	1	39	- 0.5	1.2
	1.5	37	- 0.6	1.5
	2	39	± 0	1.2
	2.5	40	- 0.5	1.2
O.F.	1	39	- 0.5	1.1
	1.5	40	- 0.5	1.0
	2	38	+ 0.1	1.0
	2.5	39	- 0.7	1.2

sidered as adequate to the extent that they corresponded to a circulation which covered the actual needs. The continuous moderate increase in pulse-rate during somewhat heavy work, which in later experiments extending over one hour usually persisted throughout the entire course of the working time, can be explained as due to the gradual onset of circulatory fatigue, diminished efficiency and increasing body temperature. The results agree with those of TAYLOR (1941) who found that the heart rate in the steady state

during exercise of 45 minutes duration continued to rise slightly throughout the work. The experiments thus show that it is important when comparing pulse-rates in different experiments, to compare values obtained after the same time from the beginning of work, a fact already pointed out by HOHWÜ CHRISTENSEN (1931).

Based on these control experiments a working time of 5 minutes was considered sufficient before the collection of expired air was commenced. As the working pulse-rate the value read after 6 minutes was taken. To determine how far such pulse-rate values are representative of the pulse-rate averages during an entire respiratory experiment, the mean of differences of these 6-minute pulse counts from the average are set out in Table 21 for the entire observational material. It will be seen that this varies between $+0.1$ and -0.7 whilst its standard deviation varies between 0.8 to 1.5.

Results

1. The relation between pulse-rate and oxygen consumption during work

For each and every day on which experiments were done during the entire course of the investigation, the relation between pulse-rate and oxygen consumption was plotted graphically, with pulse-rates as ordinates and oxygen consumption as abscissae. In assessing these graphical results it was possible, of course, to obtain a purely visual impression by comparison of the separate diagrams. But as the aim was to follow more exactly the developments during a large number of experiment days a numerical assessment was sought.

To derive this, the starting point was the rectilinear relation which exists ideally between pulse-rate and oxygen consumption. For every experiment day a line was plotted, which was satisfied by the observations according to the method of least squares, and these lines were then compared with regard to their level and slope.

Both the level and the slope are necessarily associated with a certain standard error in the separate series. It is immediately

TABLE 22. *Tests on bicycle ergometer in the morning before food intake. Pulse-rate level and angular coefficient of pulse-O₂-consumption diagrams and averages of the ventilation coefficient. The pulse-rate level is calculated for an oxygen consumption of 1.20 l/min., which is the mean for the total number of experiments. The angular coefficient is computed by taking pulse beat and litre O₂-consumption as corresponding units. Subject: E.L.*

Date	Pulse-rate level	Angular coefficient	Ventilation coefficient	Remarks
3.4	88.1±1.2	33.6±2.0	17.6	Very slight chill
20.4	87.2±0.9	39.0±0.3	22.9	
27.4	88.8±1.4	47.7±6.3	22.1	
7.5	94.5±1.7	36.3±5.3	21.8	
15.5	85.0±0.8	32.0±3.0	20.9	
28.5	107.1±2.3	40.6±7.5	24.2	
				Dance until 12 o'clock the previous night.
6.6	89.2±1.5	32.7±4.2	22.4	Chill. On the train the whole previous day.
7.6	86.7±1.2	44.5±3.5	22.5	
11.6	96.2±0.5	43.0±1.7	22.4	
19.6	83.1±0.5	33.9±1.4	21.3	
2.7	97.8±1.0	32.6±3.5	20.7	
10.7	89.2±0.4	31.7±1.1	22.8	Chill.
25.7	87.5±0.9	43.5±2.8	20.9	
30.7	84.7±2.5	33.5±7.2	20.2	
15.8	88.2±0.8	40.2±3.1	20.4	
20.8	84.6±0.7	40.9±2.5	21.5	
1.9	84.4±1.5	38.1±6.0	22.1	
7.9	83.2±0.5	27.7±1.5	21.4	Chill. Chill. Chill.
10.9	87.9±2.5	47.6±8.7	20.7	
20.9	90.3±1.0	27.0±2.2	20.2	
24.9	94.1±1.9	25.2±4.9	20.8	
2.10	89.7±1.4	28.0±4.4	19.7	
12.10	81.6±0.4	22.9±0.8	20.5	
19.10	87.0±1.2	29.4±3.6	23.3	Slight chill. Chill. Chill.
25.10	83.9±0.9	23.4±1.9	20.1	
30.10	89.2±0.4	33.3±1.0	22.8	
9.11	79.6±1.3	30.9±3.2	19.6	
15.11	82.2±0.6	29.2±2.0	19.5	
23.11	81.4±0.4	21.4±1.0	20.7	
3.12	83.1±1.0	24.6±3.0	21.5	Slight chill. Chill. Chill.
12.12	87.9±1.6	29.1±5.5	21.7	
19.12	91.3±0.7	33.1±2.7	21.4	

TABLE 23. *Tests on bicycle ergometer in the morning before food intake. The pulse-rate level is calculated for an O_2 -consumption of 1.5 l/min. Subject: J.O.*

Date	Pulse-rate level	Angular coefficient	Ventilation coefficient	Remarks
20.1	99.2 ± 0.7	17.6 ± 1.5	16.8	Walked 12 km the previous night.
26.1	98.5 ± 1.8	15.4 ± 3.7	15.5	
1.2	97.7 ± 2.6	37.5 ± 6.6	15.4	
22.2	94.0 ± 3.3	27.2 ± 8.3	17.2	
1.3	89.5 ± 0.6	33.2 ± 2.0	15.5	
15.3	91.2 ± 1.0	29.8 ± 2.1	18.7	
21.3	96.2 ± 0.7	30.7 ± 1.8	18.1	
9.4	94.6 ± 1.4	33.6 ± 1.3	18.0	
24.4	89.5 ± 1.4	25.7 ± 3.0	17.2	
2.5	90.5 ± 0.7	27.2 ± 1.5	18.6	
8.5	91.9 ± 0.7	31.2 ± 1.7	17.6	Cycled 12 km the previous night.
1.6	100.0 ± 0.4	32.9 ± 1.1	19.0	
7.6	90.3 ± 0.5	28.3 ± 1.1	17.6	
13.6	91.1 ± 0.9	25.4 ± 2.3	18.0	
21.6	92.6 ± 1.5	20.7 ± 3.2	17.1	
29.6	88.6 ± 0.8	28.0 ± 2.0	17.5	
4.7	89.2 ± 0.6	27.9 ± 1.6	17.4	
9.7	95.1 ± 0.4	30.3 ± 1.0	17.7	
6.8	92.1 ± 0.4	22.7 ± 0.8	17.1	
13.8	91.1 ± 0.6	26.4 ± 1.7	17.3	
22.8	86.9 ± 0.4	32.6 ± 1.1	18.2	Slept only 4 to 5 hours the previous night.
29.8	91.0 ± 0.4	31.1 ± 1.0	17.2	
3.9	91.6 ± 1.2	32.2 ± 2.8	16.5	
11.9	89.1 ± 0.4	29.9 ± 0.9	17.3	
25.9	91.6 ± 0.4	27.8 ± 1.0	16.8	
16.10	85.1 ± 2.0	25.7 ± 4.7	16.6	
26.10	93.8 ± 1.8	27.7 ± 4.5	17.6	
1.11	90.5 ± 1.0	26.4 ± 2.7	18.6	
8.11	90.7 ± 1.6	24.0 ± 3.1	16.1	
14.11	98.8 ± 1.4	39.9 ± 3.7	17.9	
21.11	94.4 ± 1.7	32.9 ± 4.7	19.3	Chill
11.12	90.8 ± 0.4	26.6 ± 1.8	16.1	
17.12	98.4 ± 0.9	24.5 ± 2.6	18.3	

TABLE 24. *Tests on bicycle ergometer in the morning before food intake. The pulse-rate level is calculated for an O₂-consumption of 1.58 l/min. Subject: J.S.*

Date	Pulse-rate level	Angular coefficient	Ventilation coefficient	Remarks
12.1	106.3±1.1	31.5±2.4	22.8	Chill.
17.1	100.0±2.7	30.0±5.2	22.4	
25.1	87.2±1.4	36.1±10.3	19.0	
1.2	93.5±1.0	30.3±1.9	18.6	
9.2	100.0±0.8	31.3±6.4	21.1	
23.2	100.0±1.5	25.6±2.7	22.9	
2.3	94.7±1.2	23.3±2.2	21.6	
7.3	90.7±1.1	28.0±2.4	20.3	
14.3	95.9±2.1	29.5±1.5	20.8	
22.3	92.9±2.4	30.3±6.4	20.7	Chill.
6.4	94.6±2.3	27.5±5.5	22.1	
16.4	94.1±1.4	32.3±3.5	19.9	
26.4	91.0±0.9	31.4±2.2	20.5	
4.5	96.3±1.4	34.4±3.5	21.6	Slight chill the day before.
11.5	92.6±0.6	21.4±3.7	23.0	
16.5	92.3±0.7	24.7±1.7	21.3	
22.5	99.4±1.4	30.8±3.5	22.1	
30.5	108.2±0.4	25.8±1.1	23.3	Drinking of spirits the previous night.
4.6	104.5±0.2	39.2±0.6	25.3	
12.6	94.9±0.6	29.0±1.8	20.4	
21.6	97.3±0.5	27.2±1.2	20.7	
28.6	94.9±1.7	33.9±4.5	20.1	Slight chill. Some headache.
6.7	97.3±1.4	21.8±3.3	22.3	
12.7	91.4±1.1	25.9±3.1	20.8	
7.8	97.7±0.8	28.8±2.0	20.6	
16.8	96.0±0.6	27.6±1.9	20.8	Slight chill.
21.8	94.8±1.6	31.1±4.2	20.7	
31.8	95.3±0.3	31.4±0.8	21.3	
4.9	90.9±0.9	31.5±2.4	20.4	
13.9	88.7±0.7	23.6±1.5	19.2	
17.9	91.5±1.1	27.9±2.6	19.4	
27.9	90.4±0.5	26.2±1.2	20.8	
4.10	89.8±0.3	23.3±0.6	19.4	
10.10	90.9±1.3	26.4±3.1	20.0	
17.10	92.3±1.2	30.7±2.9	21.4	
27.10	94.2±1.2	25.8±2.5	20.7	
1.11	96.8±0.7	33.0±1.7	22.6	
5.11	96.3±0.7	36.1±1.8	23.3	
12.11	93.7±1.6	29.1±3.3	22.7	

TABLE 25. *Tests on bicycle ergometer in the morning before food intake. The pulse-rate level is calculated for an O₂-consumption of 1.45 l/min. Subject: B.N.*

Date	Pulse-rate level	Angular coefficient	Ventilation coefficient	Remarks
10.1	102.9±2.0	49.3±6.1	19.0	Chill.
20.1	107.6±1.3	35.9±4.3	19.1	
25.1	99.7±1.1	33.5±2.5	17.3	
2.2	94.9±0.7	19.7±1.1	18.2	
9.2	102.3±1.9	34.4±4.2	18.7	
15.2	102.9±1.9	24.7±3.2	17.8	
22.2	108.8±2.0	26.9±5.6	20.1	
1.3	99.7±2.6	42.2±8.5	16.7	
6.3	96.5±2.5	44.8±8.5	19.9	
14.3	95.0±1.0	35.7±2.7	19.9	
22.3	96.8±1.8	29.0±4.5	17.8	
6.4	99.4±0.6	48.5±2.1	19.2	
25.4	104.5±0.6	29.6±1.6	21.0	
12.5	100.7±0.5	37.2±1.6	19.1	Athletics on previous evening.
17.5	96.0±0.9	29.1±2.5	18.6	
23.5	102.4±1.6	35.1±4.4	20.0	Enteritis acuta.
31.5	104.0±1.2	35.9±3.4	20.4	
5.6	90.6±1.1	29.3±2.4	18.2	
15.6	92.4±1.6	30.0±4.8	17.1	
30.6	98.6±1.4	41.3±4.2	17.9	
5.7	98.9±0.7	32.1±1.9	17.9	
13.7	96.9±0.5	31.1±1.4	17.9	Athletics on previous evening
6.8	104.4±1.7	39.7±5.6	18.3	
14.8	95.3±1.1	33.0±3.4	17.9	Chill. Late to bed the night before.
24.8	99.2±0.7	36.3±1.6	17.9	
27.8	103.2±1.1	34.1±3.5	18.6	
6.9	95.9±1.2	24.1±2.9	17.1	
15.9	98.0±1.9	32.6±5.4	18.6	Chill.
19.9	93.5±1.7	28.6±5.0	17.9	
28.9	94.4±0.6	30.0±1.8	17.9	
3.10	96.9±1.5	32.6±3.8	18.3	
8.10	97.2±0.4	32.6±0.9	17.5	
15.10	99.7±0.8	32.3±2.0	16.9	
22.10	92.5±1.4	32.3±3.9	18.3	
3.11	97.3±1.8	29.5±4.3	19.2	
6.11	90.3±1.3	29.9±3.5	16.5	
16.11	108.8±0.9	44.1±2.2	19.7	
20.11	100.4±0.9	32.9±2.7	19.9	
10.12	93.5±1.4	31.9±4.5	17.4	
13.12	94.8±0.9	34.2±3.1	16.9	

TABLE 26. *Tests on bicycle ergometer in the morning before food intake. The pulse-rate level is calculated for an O₂-consumption of 1.41 l/min. Subject: O.F.*

Date	Pulse-rate level	Angular coefficient	Ventilation coefficient	Remarks
23.2	112.4±0.7	42.2±1.8	18.0	Chill.
3.3	111.6±1.2	31.1±3.5	19.4	
15.3	105.6±2.0	26.8±5.6	17.8	
21.3	107.5±1.2	32.3±3.5	19.0	
5.4	124.3±1.7	35.7±4.7	18.5	
23.4	108.7±0.9	29.4±2.3	19.8	
3.5	111.1±0.9	33.2±2.8	17.7	
9.5	105.7±2.0	32.6±5.7	18.0	
14.5	112.3±1.5	31.8±3.9	17.6	
25.5	115.0±1.3	43.2±4.1	21.0	
29.5	112.3±0.4	37.0±1.2	19.5	Slight chill.
8.6	103.7±2.3	34.9±5.5	19.1	
14.6	99.5±1.0	34.3±2.7	18.0	
18.6	101.2±0.6	32.0±1.8	18.4	
3.7	108.3±0.5	36.9±1.7	19.0	
11.7	110.8±1.7	34.1±5.0	18.3	
24.7	104.0±2.4	32.4±6.4	19.6	
31.7	109.9±2.1	42.6±5.9	20.0	
9.8	115.7±0.7	33.5±2.4	18.3	
11.8	110.5±1.1	28.3±3.3	19.8	Danced until 4 a.m.
17.8	109.6±1.5	33.3±4.8	20.9	
23.8	111.6±1.6	33.6±4.8	20.4	
28.8	108.9±0.6	33.6±2.0	19.5	
5.9	107.9±0.4	24.5±1.0	18.6	
14.9	106.6±1.7	42.5±4.9	19.4	
18.9	102.8±0.5	33.4±1.3	19.1	
26.9	106.4±0.4	27.5±1.1	18.0	
5.10	110.0±1.0	28.8±2.6	21.2	
11.10	111.0±1.1	44.1±3.6	20.2	Chill.
18.10	105.2±2.2	22.3±6.5	19.0	
22.10	105.3±0.6	27.6±1.7	19.3	
24.10	108.6±1.1	38.8±3.4	20.2	
2.11	113.1±0.8	36.1±2.3	18.9	
7.11	107.7±0.4	41.8±1.1	21.4	
13.11	127.8±1.1	34.6±2.7	20.9	
19.11	113.9±0.5	47.1±1.7	20.5	
20.11	121.3±1.0	62.4±3.4	22.6	
5.12	110.1±0.9	31.1±3.1	19.5	Slept 3 hours. Arrived from Oslo in the night.
11.12	111.5±2.4	33.7±6.6	18.6	
18.12	111.8±0.7	28.7±2.2	18.4	

apparent that the error in the level of the line, expressed in pulse values for a given oxygen consumption, varies at different O_2 co-ordinates because of the error of slope. This error of level traverses a minimum for the O_2 value corresponding to the average for the total series of observations. Therefore, if the level of the line is required with the greatest statistical significance it must be calculated for the pulse-rate corresponding to this mean oxygen value. Of course this oxygen value varied somewhat from day to day. But the aim was to compare the levels for different days. Hence these levels were expressed as the pulse co-ordinate for that oxygen value which represented the mean oxygen consumption for the total number of experiments for the man concerned.

For the different men this average varied somewhat, of course, so that the pulse level in question does not permit any inter-individual comparisons. But the method was suitable for following the development in the same individual, and this was an aim of the investigation.

Table 22 to 26 give the data from the pulse- O_2 -diagrams together with their standard errors. The calculations were done as follows: Let the pulse co-ordinates be y and the O_2 co-ordinates be x .

The relation between these is thus given by the usual equation: $y = a + bx$,

and according to the method of least squares the regression coefficients are

$$a = \frac{S(y) \cdot S(x^2) - S(x) \cdot S(xy)}{n \cdot S(x^2) - [S(x)]^2} \text{ and}$$

$$b = \frac{n \cdot S(xy) - S(x) \cdot S(y)}{n \cdot S(x^2) - [S(x)]^2},$$

where n = number of observations.

The mean pulse level for an oxygen consumption of x_1 , is estimated from $y_1 = a + bx_1$,

where x_1 = the average for a total of x -observations on the subject.

According to CRAMÉR (1945), the standard error for y_1 is thus

$$\hat{\epsilon}_{(y_1)} = \pm \sigma \cdot \sqrt{\frac{S(x^2) - 2x_1 \cdot S(x) + n \cdot x_1^2}{n \cdot S(x^2) - [S(x)]^2}}$$

and for b likewise

$$\hat{\epsilon}_{(b)} = \pm \sigma \cdot \sqrt{\frac{n}{n \cdot S(x^2) - [S(x)]^2}}$$

where the standard deviation from the regression line is estimated by

$$\sigma = \sqrt{\frac{S(y^2) - a \cdot S(y) - b \cdot S(xy)}{n - 2}}$$

2. The Ventilation Coefficient

The average ventilation coefficient (ventilation of the lungs in litres per litre O_2 consumed) for the four experiments on the bicycle ergometer each experimental day is also given in Tables 22 to 26. It is justifiable to consider such an average as an expression of the normal distribution of values when the metabolic rate at the load in question has not reached the level at which the ventilation coefficient commences to rise. In order to determine whether the

TABLE 27. Test series on bicycle ergometer. Analysis of variance of ventilation coefficients within and between experimental days.

Subject	Degrees of freedom		Quotient of variances	P	Standard deviation within days
	1	2			
E.L.	31	98	6.66	<0.001	1.04
J.O.	32	96	7.00	<0.001	0.73
J.S.	38	114	8.89	<0.001	0.93
B.N.	39	115	7.11	<0.001	0.85
O.F.	39	119	8.28	<0.001	0.80

variations from day to day of these averages were significant, analysis of variance was applied. The results are given in Table 27 where P signifies the degree of probability that the variations have been at random. It is obvious that all the subjects have shown systematic differences between different experimental days.

In order to ascertain the general clinical value of this test it was further of interest to analyse the degree of variance of the ventilation coefficient in the experiments for each day which constituted the given averages. For this purpose the analysis proceeded from the intergroup variation on the basis of which the mean standard deviation for single days was computed. It will be seen from Table 27 that it varied between 0.7 and 1.0 litres.

3. The long-term trends

The averages given in Tables 22 to 26 are also shown in the diagrams of Fig. 8 to 12 where the mean pulse level and the slope of the pulse-oxygen consumption diagram for each day and the mean of the ventilation coefficient are plotted against the time axis. The

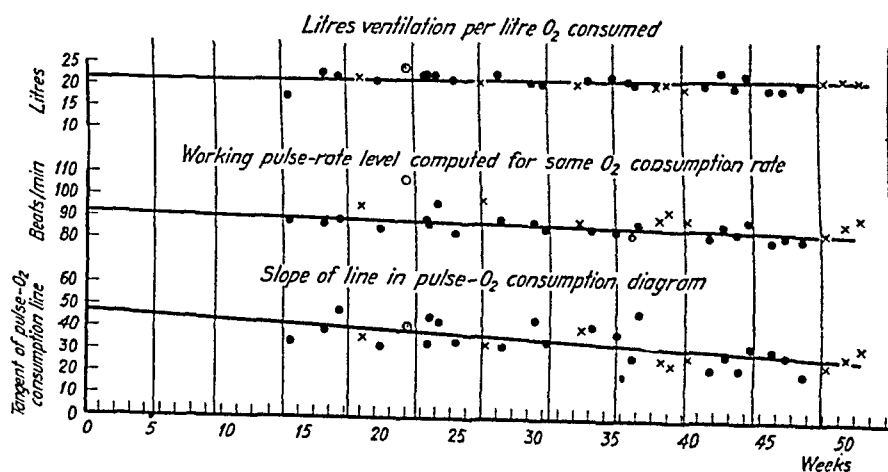


Fig. 8. Morning experiments on the bicycle ergometer during the course of the investigation. ● normal day, × chilled, ○ day after evening athletics, ○ insufficient sleep, + drinking of spirits the previous night Subject E.L.

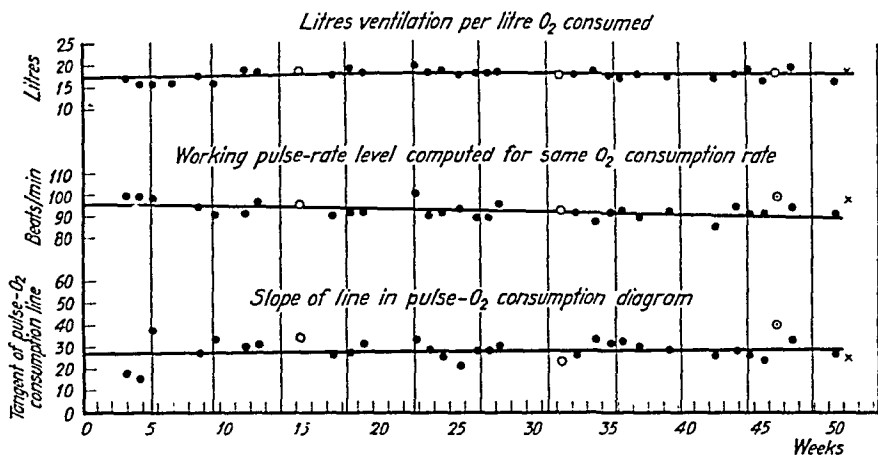


Fig. 9. Morning experiments on the bicycle ergometer. Subject J.O.

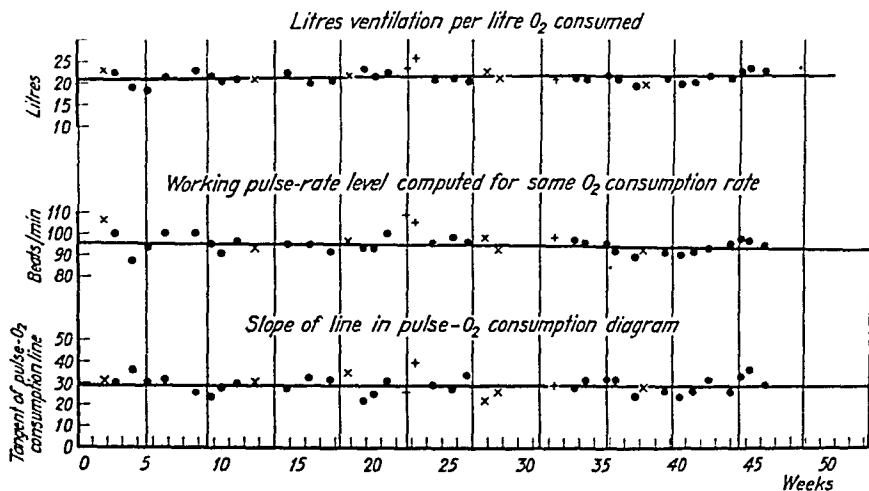


Fig. 10. Morning experiments on the bicycle ergometer. Subject J.S.

results from "normal" and "not normal" days are separately indicated.

The chief purpose of this analysis was to investigate whether there was any systematic trend of change of these factors during the period of observation. A mere glance at the "normal day" values

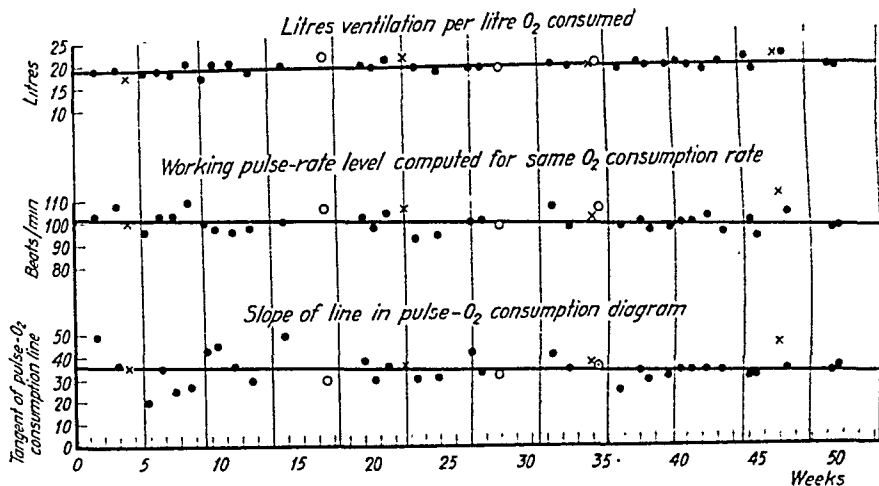


Fig. 11. Morning experiments on the bicycle ergometer. Subject B.N.

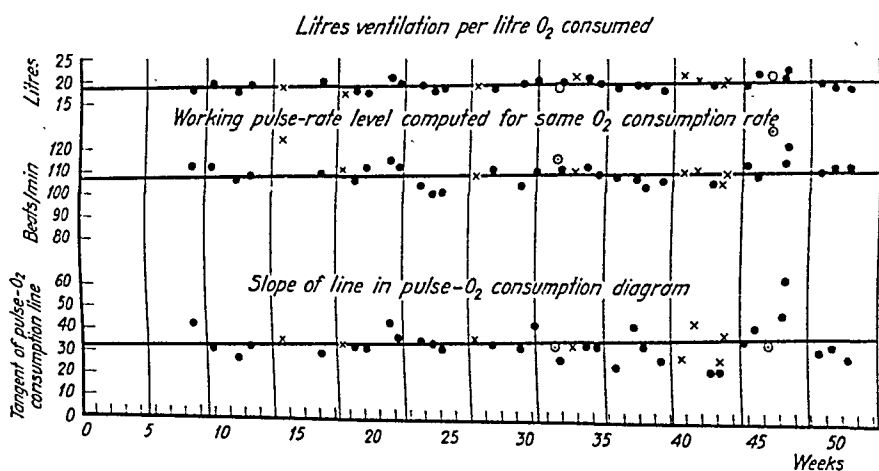


Fig. 12. Morning experiments on the bicycle ergometer. Subject O.F.

given in the diagrams shows that their regressions on the time axis can be represented as rectilinear. In view of this, the regressions for all cases have been computed and are represented by the straight lines given in the diagrams.

As a unit of time one week was used, and accordingly the

number of weeks during the year 1945 are shown along the time axis. This unit suffers from some imperfection of course, as it is not the behaviour of the factors at certain dates which is wanted but their connection with the bodily effort expended previous to these dates. This bodily expenditure is of course not directly dependent on the date as the effort varies during different days whilst holidays constitute a further source of irregularity. In spite of these sources of error it is necessary to adopt a uniform time scale, because the units of production, for example a cubic meter of firewood, are only slightly correlated with the bodily strain involved in their production and because the physiological effort measured by the metabolic rate could not of course be recorded continuously.

When computing these lines of regression the values for each single day have been given the same significance in spite of the fact that they are impaired by varying standard errors. This does not seem to imply any practically important discrepancy, however, because the variation of the standard errors is of small range only, and must moreover be considered to be caused by purely random factors. There is thus no reason to presume any systematic change of the errors occurring with the variations of the physiological factors.

The employment of rectilinear regressions involves, of course, a certain simplification in that a somewhat systematic trend in separate parts can be traced in some diagrams. Such sections, as far as they are satisfied by a fair number of points, occur only very sporadically, however, and are not very marked. Furthermore, their cause cannot be established. It thus seems unprofitable to try to produce anything but regressions of the first degree. But when this restriction is adopted the standard errors of the regressions must be looked upon rather critically as regards their capacity to express rigorously the trends of the physiological factors. In so far as curvilinear regressions occur the measures of deviation of the rectilinear correlations will be somewhat too high.

The equations of the regression lines in the different cases are collected in Table 28. To decide whether the trends express true

TABLE 28. Tests on bicycle ergometer in the morning before food intake. Normal days. Time-regressions of pulse-rate level and angular coefficient in the pulse-O₂-diagrams and the average ventilation coefficient. *t*-test of the probability that the physiological factors have shown a continuous linear trend to change. *P* denotes the probability that the lines of regression are horizontal.

Sub- ject	Mean pulse-rate level			Angular coefficient			Ventilation coefficient					
	Regression	t	Degrees of freedom	P	Regression	t	Degrees of freedom	P	Regression	t	Degrees of freedom	P
E.L.	$y = 91.77 - 0.18x$	2.82	20	0.02— 0.01	$y = 47.63 - 0.41x$	2.94	20	0.01— 0.001	$y = 21.69 - 0.01x$	0.375	20	0.8— 0.7
J.O.	$y = 95.43 - 0.12x$	2.82	27	0.01— 0.001	$y = 26.98 + 0.04x$	0.622	27	0.6— 0.5	$y = 17.14 + 0.01x$	0.750	27	0.5— 0.4
J.S.	$y = 95.60 - 0.06x$	1.38	28	0.2— 0.1	$y = 29.05 - 0.004x$	0.092	28	>0.9	$y = 20.81 + 0.01x$	0.467	28	0.7— 0.6
B.N.	$y = 101.70 - 0.14x$	3.17	31	0.01— 0.001	$y = 35.59 - 0.09x$	3.62	31	0.01— 0.001	$y = 19.03 - 0.03x$	2.55	31	0.02— 0.01
O.F.	$y = 106.94 + 0.07x$	1.10	28	0.3— 0.2	$y = 31.55 + 0.11x$	0.944	28	0.4— 0.3	$y = 18.28 + 0.03x$	2.01	28	0.1— 0.05

changes of the functional criteria, the t-test was applied. For this purpose the general formula of FISHER (1941, p. 153) can be used:

$$t = \frac{b - B}{\varepsilon(b)}$$

In the present case b indicates the angular coefficient of the regression line and B which represents the hypothetical value which is to be analysed as regards its difference from b , here becomes zero. The calculations of the coefficients and their standard errors were performed according to the formulae stated on p. 78. By entering the table of t the degrees of freedom is equal to $n - 2$.

These calculations (Table 28) gave the following results. The mean pulse-rate level of the pulse- O_2 -diagram shows that for the subjects J. O. and B. N. there was a highly probable sinking trend and for E. L. a probable sinking trend during the period of observation. The slope of the pulse- O_2 -diagram shows for E. L. and B. N. highly probable trends downwards. Finally the ventilation coefficient of B. N. shows a probable decrease. In none of the other cases can any systematic changes of any of the factors be detected by plotting linear regressions. It can thus be concluded that the tests which express the functional capacity of the oxygen transport apparatus during work indicate 'an unchanged or somewhat increased adaptation during the period of observation.

4. The normal variation range from day to day

The time-regressions for each normal days series of bicycle ergometer tests form fluent averages in which the long term changes of physiological function were eliminated as far as possible. The dispersion around the regression lines will therefore express the order of magnitude of the normal variation which can appear on different occasions without denoting any change in the physical capacity. These standard deviations (Table 29) varied between 3.1 and 4.4 for the pulse-rate level of the pulse- O_2 -diagram in different subjects; between 3.8 and 7.8 for the angular coefficient, and between 0.9 and 1.3 for the average of the ventilation coefficient.

TABLE 29. *Morning series on bicycle ergometer Normal days, fasting. Standard deviations for the time-regressions of the physiological factors.*

Subject	σ for the time-regression of		
	Mean pulse rate level in the pulse- O_2 -line	Angular coefficient in the pulse- O_2 -line	Ventilation coefficient
E.L.	3.12	6.72	1.32
J.O.	3.19	4.74	1.06
J.S.	3.27	3.80	1.28
B.N.	3.98	6.63	0.91
O.F.	4.44	7.79	1.15

5. *The co-variation between different tests made on the same days*

The determinations during resting conditions and the bicycle ergometer tests done on the same days offer a means of investigating the co-variation between the different factors which have been used as criteria for the functioning of the oxygen transport apparatus. When different variables are studied side by side during a considerable period of time, the relations between the more immediately occurring changes may differ from the relations between the slower changes, and this must be taken into account here.

For the study of the immediate changes the variate — difference correlation method (c. f. YULE and KENDALL 1937) was used. As the correlation between changes of the various factors was determined from day to day in this case, the long time trends were eliminated. The factors whose co-variation will be treated below are: the mean pulse-rate level and the angular coefficient of the pulse- O_2 -diagram, the average ventilation coefficient in the corresponding work experiments, the pulse-rate at rest and the SCHNEIDER index for the same days. As it was thought unprofitable to determine all the co-variations, the pulse-rate level at work was taken as probably the safest expression of the physical capacity and

for this reason the relation between its changes and each of the other factors was determined.

This choice of the working pulse-rate as a basis here is supported by observations on its connection with physical capacity made by previous investigators. Further, it may be mentioned that it directly indicates the reaction to a given effort and that it is not impaired by as large a standard deviation as is the angular coefficient. Among the other factors the ventilation coefficient is not quite independent of subjective influences from the experimental subject, while the pulse-rate at rest is affected by accidental influences to a relatively high degree. The SCHNEIDER index is considered too insensitive to show any higher degree of co-variation with the physical capacity.

When treating the short-term changes it is advisable that these should comprise as large a range as possible, and accordingly "normal" as well as "not normal" days of experiment were thus included.

The co-variation of the more slow changes was calculated from the absolute values of the factors. For this purpose only "normal" days were used as in this case it was specially desirable to investigate to what degree the long time trends as caused by the forestry work ran parallel with each other.

The significance of the correlation coefficient (r) was determined by means of FISHER'S t -test, according to which

$$t = \frac{r}{\sqrt{1-r^2}} \cdot \sqrt{n-2} \quad ^1)$$

The variate difference correlations (i. e. the short-term trends) are demonstrated in Table 30. This table enables the following statements to be made. The correlation between pulse-rate level and slope of the pulse- O_2 -diagram has been probable for one man only, whilst between pulse level and ventilation coefficient there was a significant correlation for two of the men and a probable correlation for one man. Between the pulse-rate level during work

¹. When calculating the t it is useful to remember that $\frac{r}{\sqrt{1-r^2}} = \tan(v)$, if $r = \sin(v)$.

TABLE 30. Correlations between changes from day to day of different tests.

δ_p = change of pulse-rate level in pulse- O_2 -diagram.

δ_b = change of angular coefficient in pulse- O_2 -diagram.

δ_c = change of average ventilation coefficient of the working tests involved in the pulse- O_2 -diagram.

δ_d = change of pulse-rate during rest in bed, fasting.

δ_e = change of Schneider index.

Correlation between	Subject					
	E.L.			J.O.		
	r	t	P	r	t	P
δ_p and δ_b	+0.12	0.65	0.6-0.5	+0.29	1.70	0.1-0.05
" " δ_c	+0.42	2.49	0.02-0.01	+0.20	1.14	0.3-0.2
" " δ_d	+0.15	0.82	0.5-0.4	+0.16	0.90	0.4-0.3
" " δ_e	-0.02	0.11	>0.9	+0.28	1.62	0.2-0.1
			J.S.			
			r t P			
δ_p and δ_b	+0.09	0.66	>0.9	+0.36	2.42	0.05-0.02
" " δ_c	+0.55	4.09	<0.001	+0.61	4.82	<0.001
" " δ_d	+0.31	2.04	0.05-0.02	+0.35	2.33	0.05-0.02
" " δ_e	± 0.00	—	1	± 0.00	—	1
			O.F.			
			r t P			
δ_p and δ_b	+0.11	0.70	0.5-0.4			
" " δ_c	+0.28	1.85	0.1-0.05			
" " δ_d	+0.42	2.92	0.01-0.001			
" " δ_e	-0.10	0.63	0.6-0.5			

and during rest the correlation was probable for two men and highly probable for one man. None of the subjects showed any correlation between the Schneider index and the pulse-rate level during work.

The long-term correlations are given in Table 31. This shows

TABLE 31. *Correlations between different tests on the same experimental days.*

p = pulse-rate level in pulse-O₂-diagram.

b = angular coefficient in pulse-O₂-diagram.

c = average ventilation coefficient from the working tests involved in the pulse-O₂-diagram.

d = pulse-rate resting in bed, fasting.

e = Schneider index.

Correlation between	Subject					
	E.L.			J.O.		
	r	t	P	r	t	P
p and b	+0.60	3.35	0.01-0.001	-0.12	0.638	0.6-0.5
p „ c	+0.39	1.90	0.1-0.05	-0.06	0.299	0.8-0.7
p „ d	+0.015	0.067	>0.9	+0.11	0.583	0.6-0.5
p „ e	+0.022	0.096	>0.9	-0.10	0.525	0.7-0.6
p and b	J.S.			B.N.		
	r	t	P	r	t	P
	+0.17	0.89	0.4-0.3	+0.31	1.82	0.1-0.05
	+0.63	4.10	<0.001	+0.46	2.88	0.01-0.001
	+0.02	0.11	>0.9	+0.62	4.30	<0.001
	+0.16	0.82	0.5-0.4	+0.07	0.38	0.8-0.7
	O.F.					
	r	t	P			
p and b	+0.56	3.55	0.01-0.001			
p „ c	+0.56	3.57	0.01-0.001			
p „ d	-0.04	0.234	0.9-0.8			
p „ e	+0.06	0.329	0.8-0.7			

that the correlation of the pulse level with the angular coefficient is highly probable for two men, that its correlation with the ventilation coefficient is significant for one man and highly probable for two men, and that its correlation to the resting pulse-rate was sig-

nificant for one man. There was no correlation with the Schneider index.

It is likely that higher correlations would be obtained with material embracing a greater number of more markedly "not normal" days. The present investigation mainly comprises days during which the functional state has changed within moderate limits only. The small changes which the different tests exhibit have thus been caused by factors occurring at random to a great extent. It may also be mentioned that the work tests were performed about one hour after the observations during rest. In investigations by some other authors where a higher degree of co-variation has been found between tests during rest and work, the former took place immediately before the work and also under different conditions, for instance in sitting position.

Changes in the Circulatory Function During Single Days

The morning experiments on the bicycle ergometer previously described, express the variations in oxygen transport function on different days. It was also of interest, however, to study the changes which occur in the course of a single day and thus to determine whether a day's work in the forest produces any signs of impaired adaptation of the circulation.

The level of circulatory efficiency and the stress imposed upon it by a given load of work depend upon constitutional factors and the general degree of training, and to a large extent upon the type of work and posture during work. Nutritional factors and fatigue from previous work also operate here. The present investigation deals principally with these effects of fatigue. It was nevertheless necessary to examine the other factors because they may interfere with fatigue effects and because, in any case, they also illuminate the same circulatory regulation problem.

Earlier Investigations

As far as the type of work is concerned, LILJESTRAND and LINDHARD (1919) found a relatively greater effect of swimming on the minute volume of the heart than other work tried. LINDHARD (1920) showed that there was a relatively higher minute volume for static than for dynamic work. COLLETT and LILJESTRAND (1924) found that the minute volume, pulse-rate and lung ventilation for a given oxygen consumption were different for different types of work such as walking, cycling and crank-turning, and also that the

levels were higher for arm work than for leg work. HOHWÜ CHRISTENSEN (1931) found similar effects. MÜLLER (1942), on the basis of his own work and his work in collaboration with GROSSE LORDEMAN (1936, 1937, 1938, 1942), considers that the smaller stress on the circulation with certain types of work arises from more favourable conditions for peripheral metabolism in such cases. The experiments of ASMUSSEN, HOHWÜ CHRISTENSEN and NIELSEN, among others, on the effects of body posture, however, show that another explanation is more probable, the higher pulse-rate with arm work being due to less favourable hydrostatic conditions for venous return than with leg work. Such an explanation also finds support in the experiments of BERGGREN (1945).

Turning now to the effects of body posture on the circulation, LINDHARD (1913) found that the heart's minute volume is less for passive standing and sitting than for lying positions. ELLIS (1921) observed that in upright positions the pulse-rate was generally increased, with lowered systolic and raised diastolic blood pressure. During the transition from lying to passive standing positions, NYLIN (1934) measured an increased difference of arterio-venous oxygen content, and increased pulse-rate and diastolic blood pressure. At the same time the systolic blood pressure, stroke volume, pulse pressure, minute volume, heart volume and the LILJESTRAND-ZANDER product all diminished.

These questions have later been brought into prominence by a comprehensive discussion by ASMUSSEN, HOHWÜ CHRISTENSEN and NIELSEN (1939). In a comparison of horizontal and passive standing positions these authors found that the latter produces a rise in pulse-rate, a lower diuretic effect of water intake, an increase of serum protein concentration and slight proteinuria. These were interpreted as signs of a certain degree of circulatory insufficiency with resulting depression of renal and intestinal function, that is to say, a compensatory vasoconstriction of the visceral vessels for the purpose of maintaining the heart's minute volume. The circulatory insufficiency is explained by plethysmographic data which showed that almost a litre of blood was deposited in the legs in passive standing positions. This hydrostatic deposition also occurred with arm work.

In further experiments the authors varied the circulating blood volume by compression cuffs on the legs, applied sometimes in states of large and sometimes in states of small venous filling. Arm work in the sitting position with legs emptied of blood yielded a large minute volume and low pulse-rate. When the legs were allowed to fill with blood, however, there was a 30 % lower minute volume and high pulse-rate. The latter is interpreted as an expression of a regulatory mechanism associated with the central venous pressure, and together with the small stroke volume it is regarded as a sign of lowered accommodation range of the circulation. The oxygen consumption was approximately the same in both cases, whilst the arterio-venous oxygen difference was larger when the circulating blood volume was less.

BRUN. KNUDSEN and RAASCHOU (1945) confirm the finding that passive standing positions diminish diuresis, and consider that the effect is due to extra-renal haemodynamic factors of the type described above together with an efferent intra-renal arteriolar constriction.

The experiments of NIELSEN and FRIDRICHSEN (1938) show that the hydrostatic factor also operates in the circulation when leg work is involved, the working pulse-rate being higher when the legs were vertical than when horizontal.

The blood displacement in different positions was examined by ATZLER and HERBST (1923), who found a larger foot volume after standing than after sitting. Such displacements are also revealed by changes in the vital capacity of the lungs. BOHR (1907) found that this is diminished by transition from standing to lying positions. According to HAMILTON and MORGAN (1931) and ASMUSSEN, HOHWÜ CHRISTENSEN and SJÖSTRAND (1939) this effect may be ascribed to an increased quantity of blood in the lungs. Further HAMILTON and MAYO (1944) observed that the vital capacity is less when the body is immersed in water to the nipple line than when the subject is standing in air.

The connection between fatigue and hydrostatic effects on the circulation is to some extent clarified by experiments on dogs by HORIUCHI (1928) where diminished blood supply to the brain was accompanied by earlier onset of fatigue. These questions are

further dealt with by HELLEBRANDT, BROGDON and TEPPER (1940) on the basis of energy metabolism measurements in different body postures. It may also be mentioned that experiments on dogs by MAYERSON (1942) indicate that circulatory failure in upright positions is primarily due to weakness on the venous side.

Nutritional effects on the circulation have been studied by COLLETT and LILJESTRAND (1924), JARISCH and LILJESTRAND (1927), v. EULER and LILJESTRAND (1927) and APÉRIA and CARLENS (1931). The circulation rate was found to be increased after meals, the increase persisting some hours after a large meal and being especially noticeable after intake of protein.

Literature is scarce on the subject of the reaction of the circulation to fatigue produced by previous work. BERGGREN and HOHWÜ CHRISTENSEN (1943) found an increased pulse-rate for standard work on such days as the subject had performed vigorous athletic exercise several hours earlier. The raised pulse-rate level was ascribed to a diminished stroke volume.

On the basis of these observations the present investigations on the relation of working pulse-rate levels to oxygen consumption were done partly in the morning and partly in the evening on ordinary working days. As a control corresponding experiments were done during resting days and on days with a more standardised occupation between the tests, namely walking in the country over fixed routes.

As described below, an increased pulse-rate level could appear during working days, and accordingly it became of interest to ascertain the cause of this. The effect could conceivably have its origin in a direct cardiac reaction of the stress of the working day. Another explanation is also possible, namely, augmented deposition of venous blood in the dependent parts of the body because of vascular dilatation or slackness of the skeletal muscles. If such is the explanation the increased working pulse levels should be of the same sort as the circulatory effects related to upright body postures. To decide this question it is of help to determine how far the increased pulse levels could be influenced by minimising the possibilities of blood collection in dependent regions, a result achieved with elastic bandages in the experiments now to be described.

Methods

The experiments were done in series with loads increasing in the same way, as already described, together with fasting tests on the bicycle ergometer. The series comprised both morning and evening experiments separated by a working or resting day.

The test work consisted partly of work on the bicycle ergometer, and partly of wood-sawing in constant bodily position. Thus they included both arm and leg work. In work on the same day, however, the same type of work was used throughout. With wood-sawing the man himself increased his work intensity from one experiment to the next by raising his speed of working and the pressure on the saw, but during each separate experiment he endeavoured to maintain a constant work intensity.

Collection of expired air and the pulse counts in the bicycle ergometer experiments were done as described previously. During wood-sawing the pulse-rate was at first measured on the lobe of the ear with a photo-electric pulse counter (MATTHES, 1941; MÜLLER, 1943; LEHMANN, 1944), the technique employed being that given by LUNDGREN and ZOTTERMAN (1945). It was soon found, however, that after the test work had been standardised the carotid pulse could be counted by hand during the work after some practice. This more simple method was subsequently used. Counting was done at half minute intervals during the entire respiration experiment beginning immediately after the commencement of collection of expired air. The working pulse-rate was taken as the average of all the counts.

Leg binding was done with ordinary elastic bandages from feet to inguinal region. The bandages were applied after the subject had rested for several minutes in a horizontal position with the legs elevated, the same position being kept during the bandaging. The bandaging was rather loose, partly because tight application inconvenienced the man when working, especially when leg work was involved, and partly because loose binding seemed to give the most marked effect on the working pulse-rate.

Results

Control experiments for factors outside work: As was seen from the review of the literature, a certain degree of stress on the circulation has to be reckoned with immediately after a meal, this even being recognisable as an increased pulse-rate level during work (JARISCH and LILJESTRAND 1927). To determine the effect of actual lumber work it was thus necessary to find how the experiments could be done so as to exclude the meal time factor.

The effect of the morning meal is shown in Fig. 13 where it will be seen that the subsequent pulse-rate level definitely rises. Accordingly the series of tests were subsequently carried out with the subject fasting.

The evening series of tests were carried out immediately before

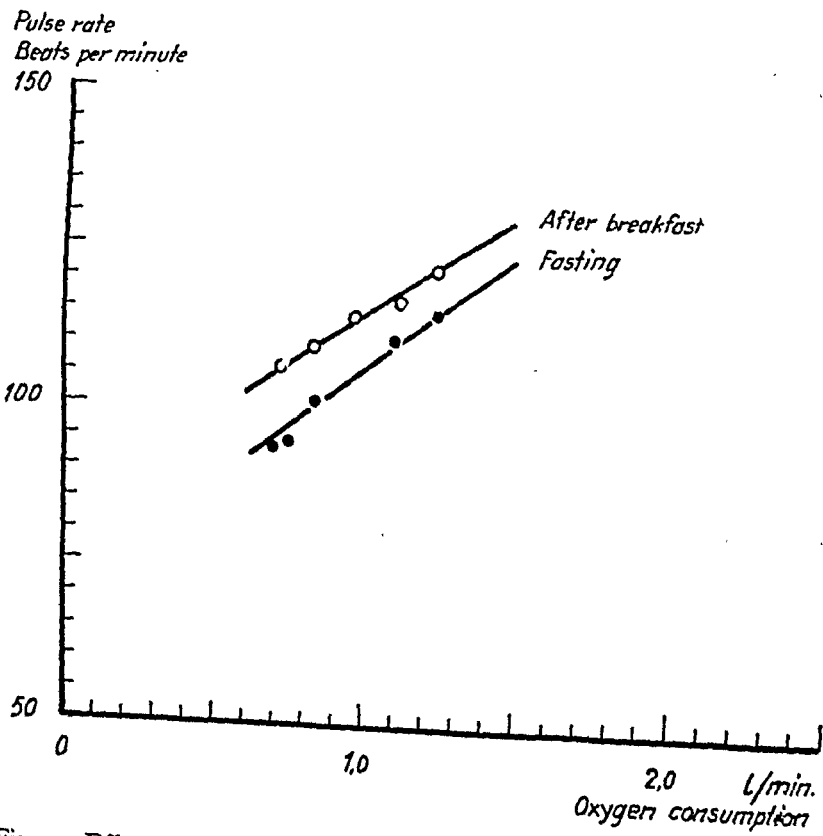


Fig. 13. Effect of breakfast on the pulse- O_2 -diagram during cross-cutting.

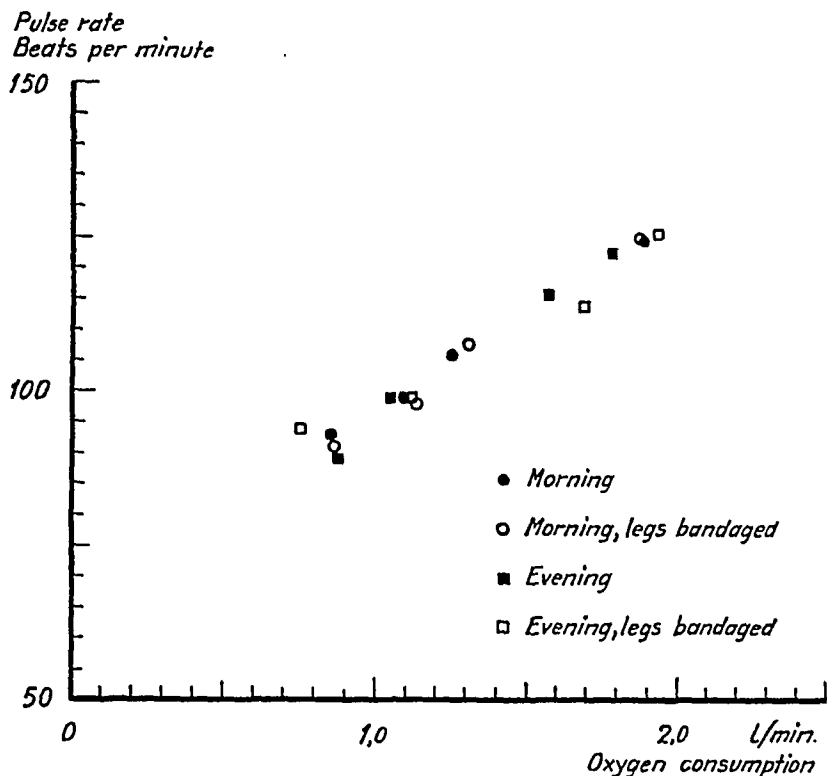


Fig. 14. Pulse-rate — oxygen consumption ratio during work on the bicycle ergometer with and without legs bandaged. Resting day.

the evening meal, at least 3 to 4 hours after the foregoing meal. To control any influence even of this foregoing meal, experiments were done on resting days with mealtimes unaltered. Rest during such days was relative, but the subject never worked in the forest and merely occupied himself by helping in the laboratory. Controls were done so that he ate approximately the same amount at mealtimes during these days as he was accustomed to eat on working days in the forest. It was then found (Fig. 14) that during these resting days the pulse-rate level was the same in morning and evening experiments both with and without leg bandages. The daily meals were thus without influence on the evening pulse level. Indeed, such an influence was unlikely because of the long interval between the mealtime and the evening experiment. It also follows

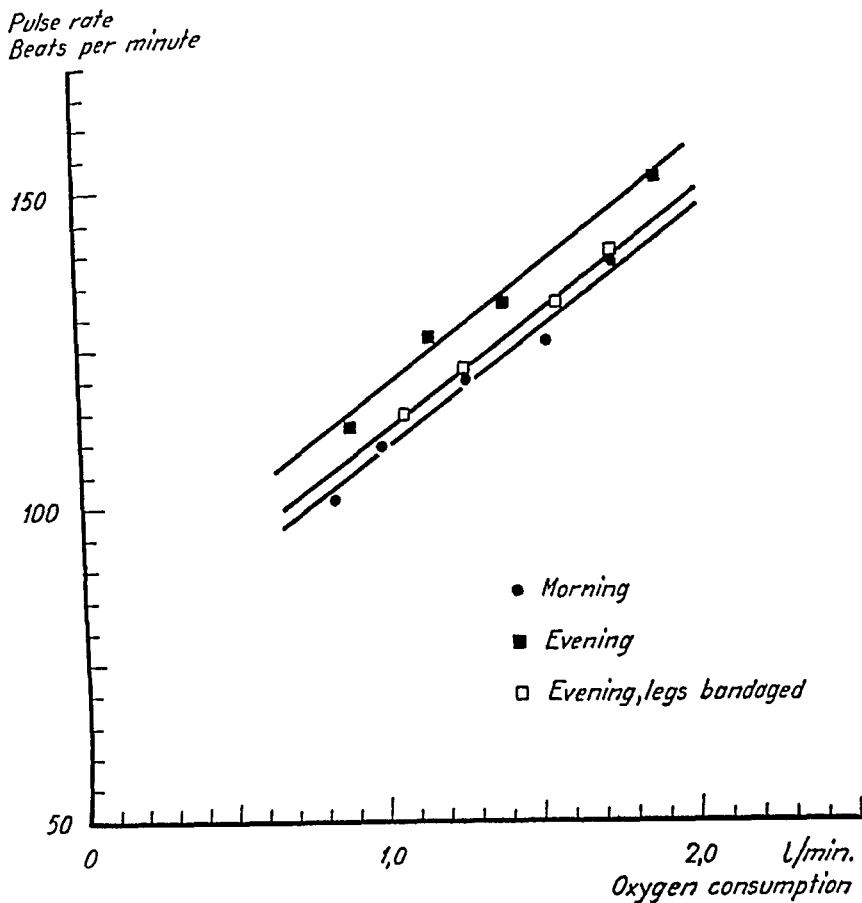


Fig. 15. Pulse- O_2 -diagrams during work on the bicycle ergometer with and without legs bandaged. Working day in the forest.

from these experiments that no other factor, such as the time of day or being occupied with minor tasks, had any effect.

Working days in the forest: During a number of experiment-days for ordinary lumber work the pulse-rate levels were the same both for the morning series and the evening series, just as in the experiments on resting days. No measurable change in the adaptation of the circulation was therefore disclosed here by the method in question. During other days on the contrary there was a raised evening level. Comparison with the results on resting days shows that this is caused by the lumber work during the day.

Pulse rate
Beats per minute

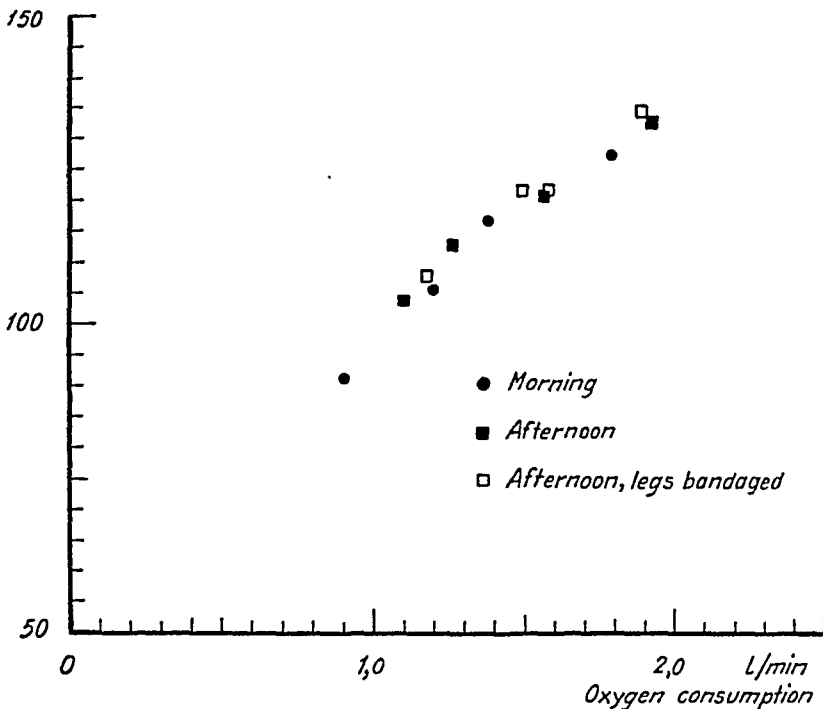


Fig. 16. Pulse-rate and oxygen consumption during work on the bicycle ergometer before and after walking 7 km. Subject O.F.

To determine the nature of the pulse-rate increase the effect of leg bandaging was now examined. In some cases this brought about a reduction of the evening pulse-rate towards the morning level, indicating that the typical evening increase arose at least partly from hydrostatic blood displacement to the dependent parts of the body (Fig. 15).

This effect of bandaging admittedly did not always appear. In such cases as it did not occur the existence of hydrostatic factors causing the evening pulse-rate rise, cannot be excluded, however. The bandages may not have been applied properly, as it was, of course, very difficult to perform the bandaging identically in different experiments. Nor was any correction possible during the course of the experiments. The bandages had to be applied in the

Pulse rate
Beats per minute

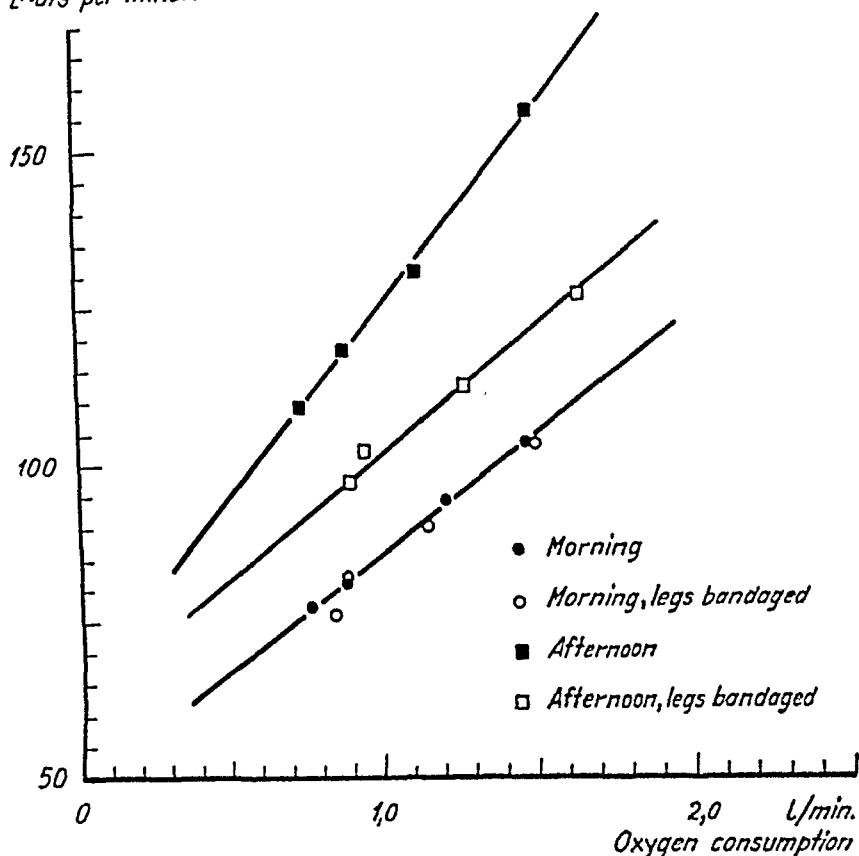


Fig. 17. Pulse-rate and oxygen consumption during work on the bicycle ergometer before and after walking 15 km. Subject O.F.

shortest possible time in order that similar conditions should be attained in the series with and without the bandages. Moreover, the result could not be foreseen before the experiment was completed and the readings computed.

In the morning experiments leg bandaging was without effect except in the case referred to below. Therefore the hydrostatic effects on the pulse-rate shown in evening tests may be ascribed to the day's work in the forest.

The effect of leg bandaging was most marked in the subjects O. F. and E. L., whilst it never appeared in J. O. With B. N. there

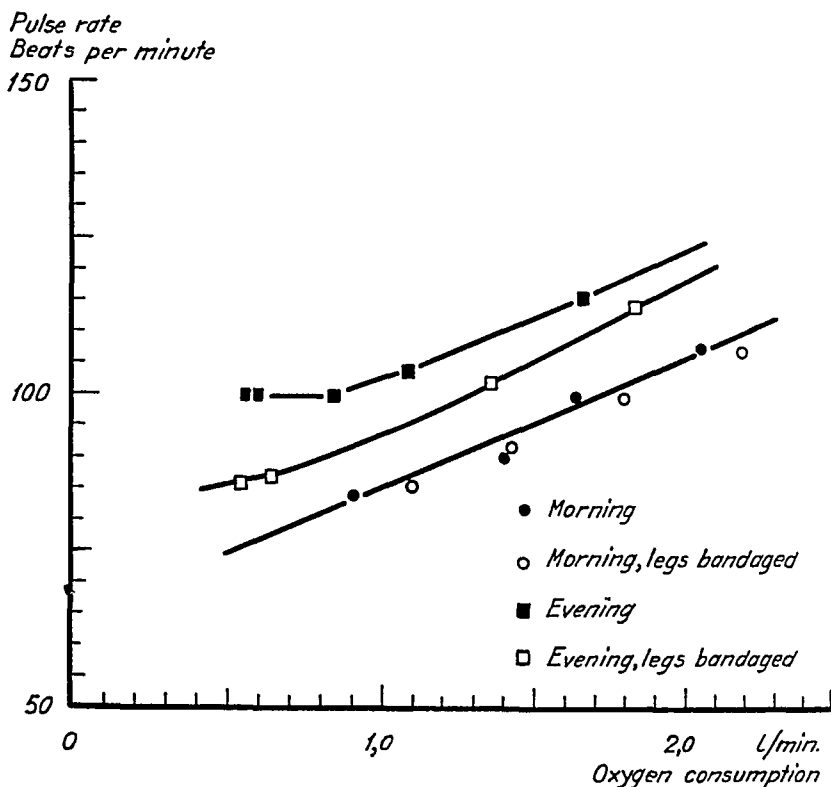


Fig. 18. Pulse-rate and oxygen consumption during cross-cutting before and after a working day in the forest. The subject (J.S.) was chilled.

was never any clear effect, but in this man there was only exceptionally any definite increase in the evening pulse-rate level. This may be related to the fact that he seldom showed any inclination to work really intensively in the forest.

Effects of cross-country marches: Lumber work is not ideally suitable for interposition between morning and evening tests because the amount of work involved varies from day to day and cannot be measured with any degree of certainty. For investigation of the quantitative relationships between fatigue-loading and pulse-rate increase therefore, a more controllable type of work was used, namely cross-country marches with approximately the same speed over distances of 7 and 15 km. The results of this are given in Figs.

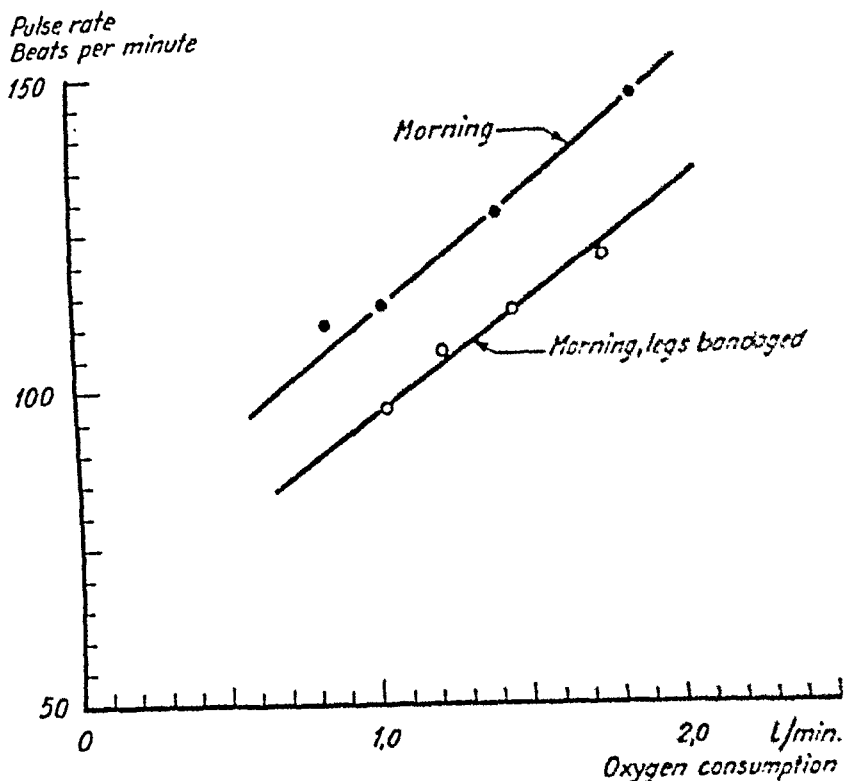


Fig. 19. Pulse-rate and oxygen consumption during work on the bicycle ergometer with and without legs bandaged. Morning after return from a strenuous journey. Subject O.F.

16 and 17 for the subject O. F. It is seen that after the shorter walk no rise of pulse-rate appeared. After long distances on the contrary the working pulse-rate increase and the effect of bandaging were both pronounced.

Effect of factors outside the work which impair physical condition: A "chill" had an accentuating effect both on the degree of evening pulse-rate increase and on the magnitude of the leg bandaging response. Fig. 18 shows the results of a working day for the subject J. S. at a time when he had an infection of the upper respiratory passages but carried out a good day's work in spite of this. Normally he showed only insignificant reactions but during this condition he had a marked pulse-rate increase in the evening

series and a definite reduction with leg bandaging. Furthermore it may be mentioned that even the resting pulse-rate in a standing position then showed differences in the evening, being 88 when legs were bandaged, increasing to 99 when the bandages were removed.

Only in one case did leg bandaging cause a fall of the morning working pulse-rate (Fig. 19). This experiment was done on O. F. the morning after he returned from a three days visit to Oslo during which he slept very little and went on intensive sightseeing. He returned in the middle of the night after a strenuous journey and had slept only three hours before the experiment. As will be seen, the pulse-rate level without leg bandaging was very high, and leg bandaging had a marked effect in lowering it. The levels found after bandaging were approximately the same as the man usually showed in normal morning experiments. It would have been of interest, of course, to do evening tests on this day too, but this was impossible because of the man's understandable reluctance to work.

Discussion

The experiments described above show that daily work in the forest sometimes causes at the end of the day a rise in the working pulse-rate level in relation to the oxygen consumption, and that this arises in part hydrostatically from blood deposition in the vascular beds of the lower extremities. Further, it has been shown that the condition is dependent on the load of work and is accentuated by a "chill" and other factors detrimental to the man's general condition.

In all probability the rise in pulse-rate is to be regarded as an active compensation for the diminished stroke-volume, so that the heart's minute volume is adjusted to give an adequate level of oxygen supply. It could even be interpreted as an expression of raised minute volume arising the suppression of oxygen consumption associated with impaired peripheral utilization. But as HOHWÜ CHRISTENSEN (1931) has shown that there is a close relation between minute volume and oxygen consumption such an explanation is scarcely probable.

The fact that the pulse increase at least partly arises from im-

paired conditions for the venous return reinforces the view that it is to be interpreted as a compensation for the maintenance of the minute volume and is not aimed to increase this. It is rather more likely in the present case that a certain degree of circulatory insufficiency existed, of the same kind as that demonstrated by ASMUSSEN, HOHWÜ CHRISTENSEN and NIELSEN during passive standing, i. e. diminished minute volume and increased peripheral utilisation.

The raised pulse-rate level as well as its return after leg-bandaging appeared both with experiments on the bicycle ergometer and with wood sawing. This agrees with the experience of previous authors on hydrostatic positional effects in arm as well as leg work. Any comparison of the magnitudes of the reactions in these two cases would be unsafe, however, partly because of the difficulties of exact measurement of the degree of strain previously in the day and partly because the leg-bandages may have been applied differently in different experiments. Further, as the morning experiments on the bicycle ergometer show, there are variations in the functional state from day to day of lumber workers, so that any comparison between the effects of leg-bandaging on arm and on leg work would require a much larger series of experiments than these.

Although the leg-bandaging was without effect in the morning series either with arm or leg work, there was nevertheless a distinctly higher pulse level with arm work. This shows that the leg-bandaging method was not sufficiently sensitive to influence the degree of blood displacement, which according to earlier authors is the cause of this difference between arm and leg work.

In the leg-bandaging experiments where there was a return of the pulse-rate in the evening experiment it was typical to find signs of arrhythmia in such a manner that periods of less and more frequent pulse beats alternated. It is possible that these are associated with the fact that the leg bandages during stronger muscle tensions of the legs give rise to fluctuating increases of venous return. The phenomenon was not analysed any closer, however.

The cause of the impaired hydrostatic condition in the evening experiments may lie in a changed vasomotor tonus or a diminished pressure exerted by the skeletal muscles on the veins. In this connection it should be mentioned that in bodily fatigue a diminished

muscle tonus has been demonstrated by RYAN, JORDAN and YATES (1919). It was also found by NOYONS (1908), GILDEMEISTER (1914) and SPRINGER (1914) that in such a condition the muscle hardness was lowered. ARNOLD and WUNDERLICH (1932) and ARNOLD (1935) found that fatigue diminishes elasticity of connective tissues.

functional capacity in sprint-work as well as in more protracted types of work.

In professional work the body temperature has been examined by, among others, STRAUSS (1931) and SIMONSON and DOBRIN (1932—33), the aim being to study the degree of adaptation to work in hot environments. Another practical application of temperature measurements has been reported by BERGGREN (1945), who used the relation between the body temperature and the metabolic rate to measure the energy output in different branches of athletics. These lines of thought have also been followed in the present investigation where the applicability of the method has been tested for computing the metabolic rate in different forms of lumber work.

Methods

The rectal temperature was measured by means of a mercury thermometer of the usual type immediately after cessation of work. The error of method has been given previously in the section on the temperature during rest. Table 32 shows a control experiment giving the course of the rectal temperature change after cessation of work which in this case consisted of one hour's cycling at a rate of 720 kg.m./min. It will be seen that 6 minutes after the work has ceased there is a fall of $0^{\circ}.1$ C. From this it is apparent that the determinations have to be made immediately on cessation of work if an expression for the body temperature during actual work is to be obtained.

The working-temperature experiments consisted of measurements during the course of the working day in the forest, each measurement being preceded by at least one hour of continuous work. Control tests were done on the bicycle ergometer using a series of loads on the same subjects in the mornings as well as in the evenings, and at different seasons of the year. The period of work in each experiment was one hour, during which the pulse-rate was counted every fifth minute whilst the respiratory exchange was determined four times in the majority of cases, although sometimes

TABLE 32. *Rectal temperatures at various times after cessation of work on the bicycle ergometer. One hour's work, 720 kg.m. per minute.*

Minutes after end of work	Rectal temp. °C	Minutes after end of work	Rectal temp. °C
1	37.68	47	36.78
6	37.58	50	36.78
10	37.42	53	36.73
15	37.36	56	36.73
20	37.25	59	36.68
25	37.14	62	36.68
30	37.08	65	36.63
35	36.98	68	36.63
38	36.92	71	36.63
41	36.88	74	36.63
44	36.85	77	36.63

this was done twice only. These experiments were intended to control the degree to which different seasons and time of the day affect the morning temperature and also to give some idea of the inter-individual variation.

Results

Control Experiments on the Bicycle Ergometer

In order to investigate the working temperature level at different times of the day, experiments involving one hour of continuous work were made in the morning between 7 and 9 o'clock as well as in the evening between 3 and 5 o'clock. Individual differences were found. Thus the subject O. F. (Fig. 20) showed the same temperature level in relation to the oxygen consumption both at morning and evening, whilst B. N. (Fig. 21) showed on the average 0°.3 C and J. O. (Fig. 22) 0°.4 C higher working temperature in the

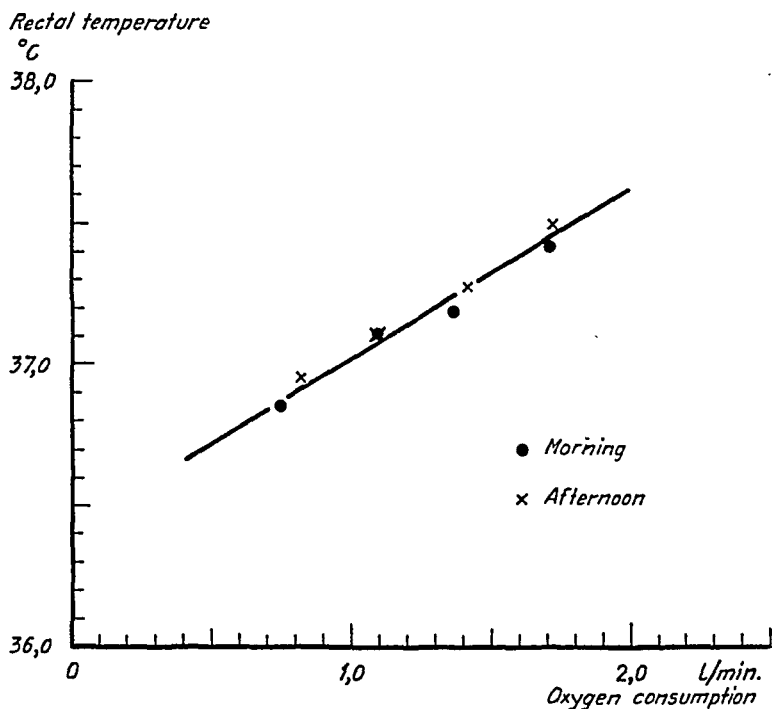


Fig. 20. Rectal temperature after one hour's work and oxygen consumption. Morning and evening experiments on the bicycle ergometer. Subject O.F.

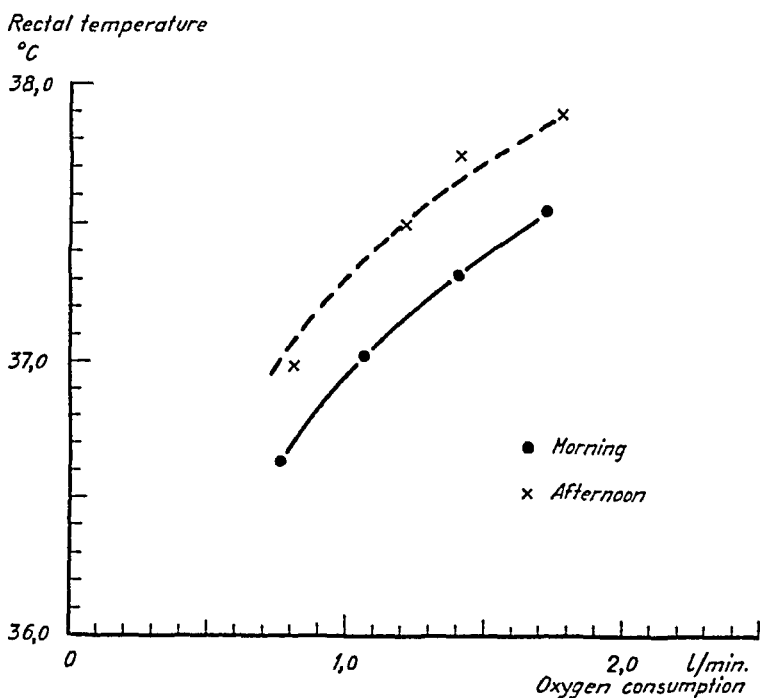


Fig. 21. Rectal temperature after one hour's work and oxygen consumption. Morning and evening experiments on the bicycle ergometer. Subject B.N.

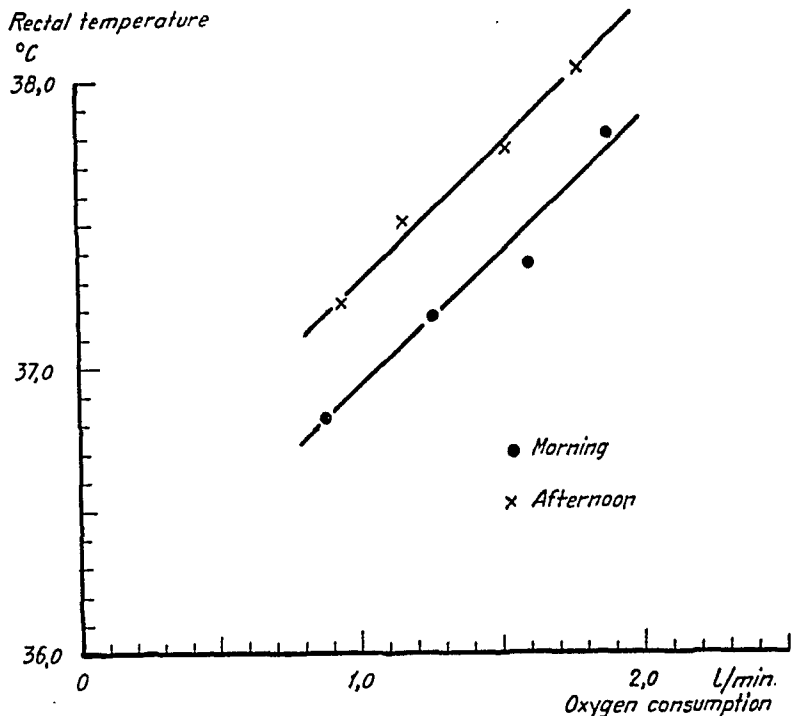


Fig. 22. Rectal temperature after one hour's work and oxygen consumption. Morning and evening experiments on the bicycle ergometer. Subject J.O.

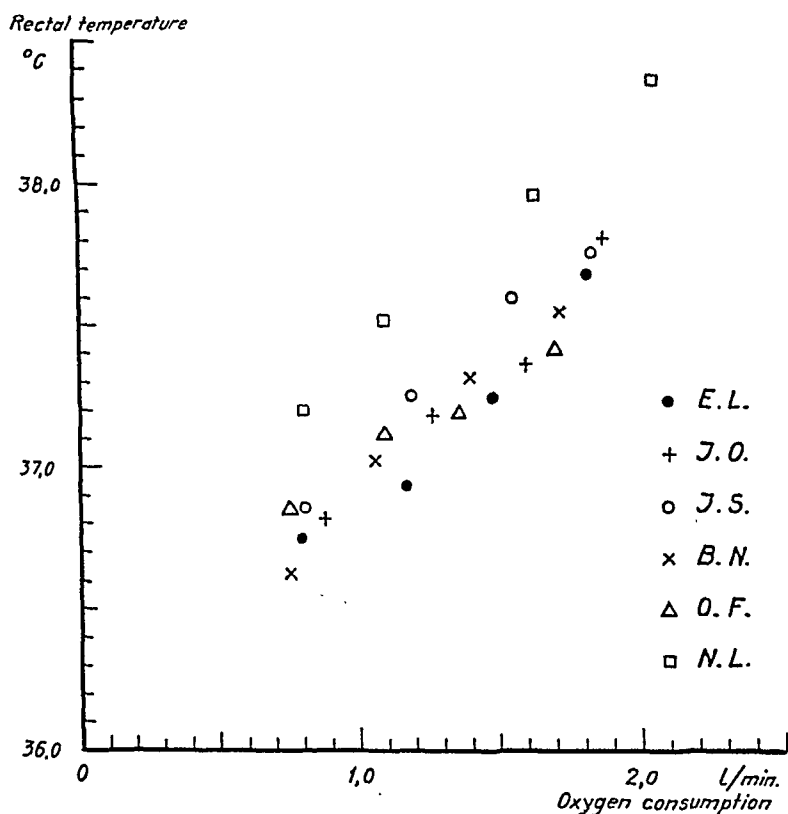


Fig. 23. Rectal temperature and oxygen consumption during work on the bicycle ergometer. Morning experiments of one hour's length on the lumber workers and a physically untrained subject (N.L.).

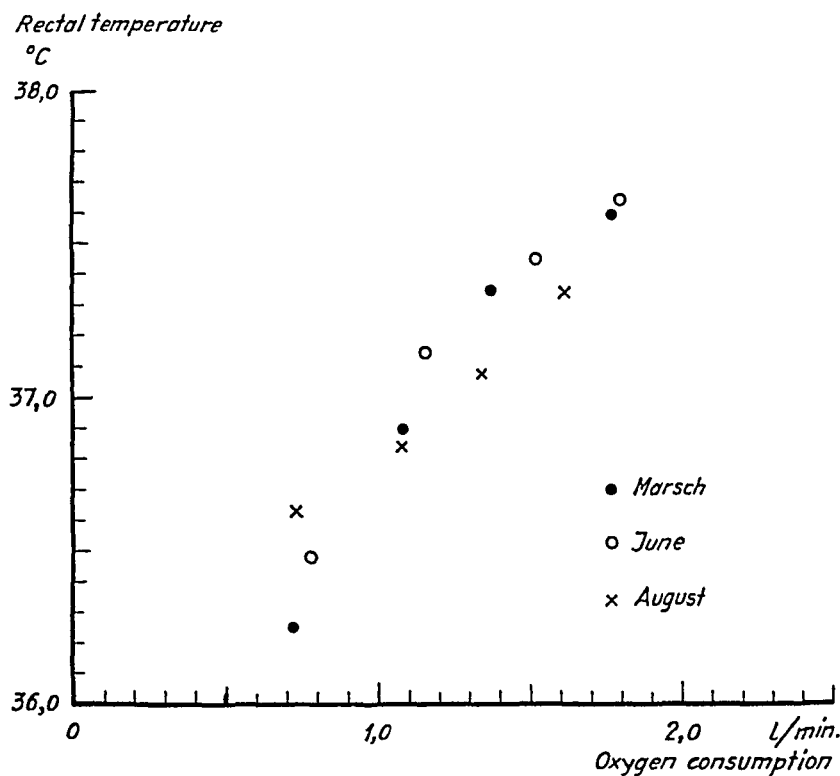


Fig. 24. Rectal temperature and oxygen consumption during one hour's work on the bicycle ergometer at different seasons, Subject B.N.

evening. The values for the other two subjects fall between these extremes.

In order to demonstrate the individual differences, the means for all morning experiments on all the five subjects have been collected in Fig. 23. Further experiments on a rather untrained person (N. L.) have been added here. It will be seen that the temperatures of the lumber workers lie at about the same level, whilst N. L. has a clearly higher working temperature. Nothing definite can be said about the cause of this difference but it is possibly associated with the different state of physical training. Differences in body size may be the cause of different body temperatures during work, as has been shown by ROBINSON (1942). But in the present case such a cause may be excluded. It may be mentioned further-

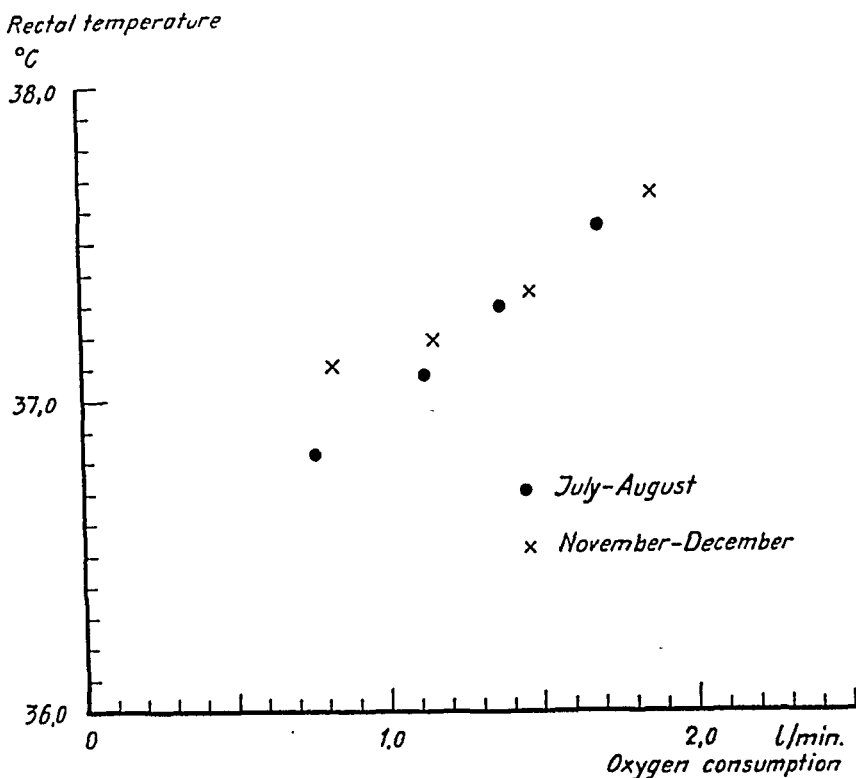


Fig. 25. Rectal temperature and oxygen consumption during one hour's work on the bicycle ergometer at different seasons. Averages for three subjects.

more, that the lumber workers' working temperatures closely correspond to those of a well-trained athlete as reported by BERGGREN (1945). A closer analysis of the correlation between the degree of training and the temperature level during work could suitably be made upon the same subject during the course of a training period but this was not done in the present investigation.

In order to ascertain whether the working temperature is affected by the season of the year, morning determinations for one subject have been collected in Fig. 24 for March, when it was still winter at the place of investigation, and for June and August. It is evident that there is a certain range for single determinations but no systematic influence of the seasons. To examine the problem more closely experiments from July—August and November—December

have been collected in Fig. 25 where every value in the diagram represents the mean of a morning and an evening determination for each one of the three subjects E. L., B. N. and O. F. It will again be seen that during higher loading in every case there is no systematic seasonal influence on the working temperature.

Experiments in the Forest

The experiments in the forest involved determinations on all the five subjects during a large number of days in order to obtain a relative expression for the metabolic rate when doing different kinds of lumber work during various seasons, days of the week and times of the day. The full results of these experiments will be published separately as they are of special interest for the work studies. Here only a few examples will be given in order to demonstrate the applicability of the method for practical purposes.

Fig. 26 shows the body temperature at different hours of the day whilst cutting timber, each value representing the mean of deter-

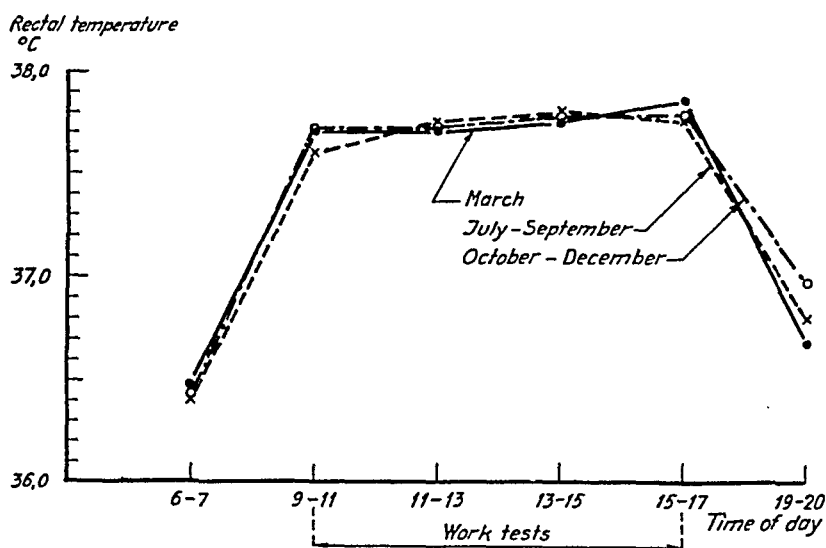


Fig. 26. Rectal temperature during cutting of timber and pulpwood. Different times of the day and different seasons. Subject O.F.

minations on several days. It will be seen that the three curves for March, July to September, and October to December, show very close agreement and that the body temperature during work changes very slightly during the day. As the subject concerned here (O. F.) had the same temperature level in the morning as well as in the afternoon during the bicycle ergometer tests, it is thus obvious that the mean metabolic rate varied within narrow limits during the working day in the forest.

Fig. 27 gives the results from cutting firewood during July to September and during November. Even here there is no difference between the seasons and by comparing this diagram with the previous one a very close agreement will be found for the temperature level of the body during work. In Fig. 27 the results for Saturdays are given separately. It is seen that these coincide with the levels of the other days.

As the bicycle ergometer tests described above did not disclose any significant seasonal variation of the ratio of work temperature to oxygen consumption, the results from this man thus lead to the following conclusion: Timber felling and firewood cutting are done

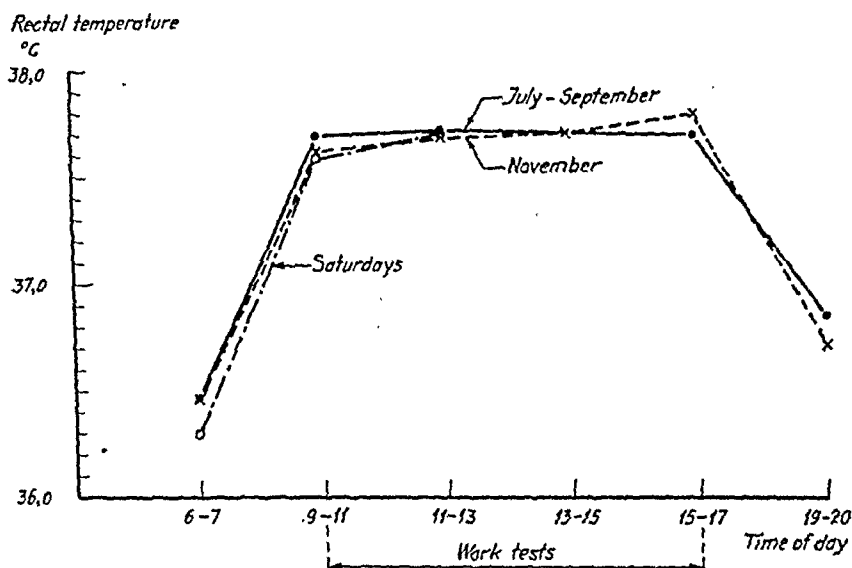


Fig. 27. Rectal temperature during firewood cutting. Different times of the day and different seasons. Saturdays taken separately. Subject O.F.

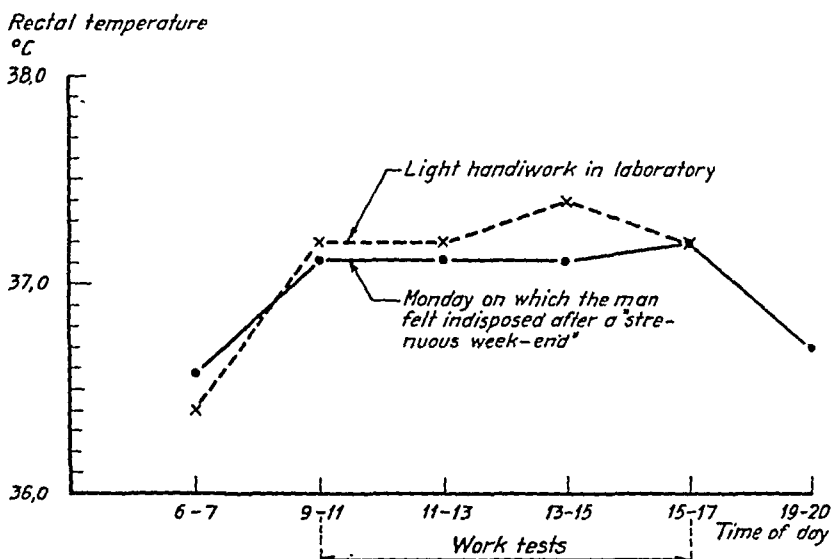


Fig. 28. Rectal temperature during light work. Subject O.F.

with approximately the same mean metabolic rate. This metabolic rate is the same during summer and winter and it is not affected by the fewer working hours on Saturdays. It must be pointed out, however, that these practical results are only valid for the person in question and are not meant to indicate a general state of affairs.

As a further demonstration of the practical use of the method, Fig. 28 gives the temperature level during days of light odd jobs as well as during a Monday when the subject was tired and did not work fully. It shows that the temperature level and thus the metabolic rate in these cases was considerably lower than during the normal working-days in the forest.

It may be mentioned, moreover, that one of the subjects whose working temperature had just been measured to be $38^{\circ}.2$ C was caught in heavy rain. After sitting wet for 45 minutes in the rest hut which was not heated his temperature had sunk to $36^{\circ}.15$ C. This shows that unfavourable conditions may lead to a fall in temperature down to subnormal values of the same kind as described by LILJESTRAND and MAGNUS (1922) for baths.

Suitability of The Working Pulse-Rate for the Determination of Energy Metabolism in Field Experiments

BERGGREN (1945) working in HOHWÜ CHRISTENSEN's laboratory pointed out the possibility of using the working pulse-rate for the determination of energy output during athletics etc. For this purpose the subject is calibrated by constructing a pulse-oxygen consumption diagram from treadmill or bicycle ergometer data. From this diagram it is then possible to read the oxygen consumption corresponding to the pulse-rate recorded during athletics.

As this method would constitute a considerable simplification of the tedious routine determinations of energy output in industrial work it was examined further as to its practical suitability for field research.

Methods

The investigation was done at the same time as another series of experiments for the purpose of measuring the energy output at different phases of firewood cutting in the forest, the general results of which will be published elsewhere. These experiments involved measurement of the respiratory exchange during a test period during work of from 3 to 7 minutes preceeded by a working period of 5 minutes. The working pulse-rate was counted concurrently. As it was impossible to palpate the pulse during this actual work, and as the use of the photo-electric pulse counter would have disturbed the men in this particular work and so render the energy output measurement unrepresentative of normal lumber work, the

pulse-rate in the immediate post-exercise period was used. According to COTTON and DILL (1935) this falls very little during the first 10 seconds after the cessation of work. In the present case the counting was done in the following way.

While the work was in progress the examiner kept close behind the workman with his hands in readiness to palpate the carotid pulse. When the word to stop work was given, the workman immediately stood upright and the time for the first ten pulse beats while standing, resting, was taken with a stop-watch. Immediately afterwards the man resumed work. This was done twice during the course of the respiration experiment and then the third and final pulse count was made after the respiratory sampling was complete. The average of these three counts was taken as the working pulse-rate. The two interruptions in the respiratory experiment of course led to a somewhat low value for the oxygen consumption but the interruptions were so short in relation to the total period of air sampling that the error could be neglected.

The applicability of the "10-beat value" to express the true working pulse-rate was controlled in a separate series of experiments on the bicycle ergometer. In these the pulse-rate during actual work was compared with the values found by measuring the time for the first 5, 10, 15 and 20 beats after cessation of work. The oxygen consumption per minute here varied between about 1.0 and 2.5 litres. The results are set out in Table 33. It will be seen that the means of differences in the 5 and 10 beat counts from the true working pulse-rate are not significantly different from zero, whilst the 15 and the 20 beat counts show a clear decrease below the true working pulse-rate. As the 10 beat counts have a lower standard deviation than the 5 beat counts the former were used in subsequent forest experiments. It follows furthermore from the standard deviation that the individual measurement is rather uncertain so that for practical use in the forest the method requires a relatively large series of determinations. This is, however, quite easy to do by the simple method described.

The average oxygen consumption obtained from respiratory measurement in the forest at various phases of work were now compared with those computed on the basis of the pulse-rate re-

TABLE 33. *Deviation of the pulse-rate early in recovery, from the working pulse-rate in experiments on the bicycle ergometer. The former has been calculated from the time for the first 5, 10, 15 and 20 pulse beats after cessation of work.*

Number of beats after cessation of work	Number of counts	Mean of differences	Standard deviation of the mean of differences
5	37	— 2.3 ± 1.3	8.2
10	35	— 2.5 ± 1.0	6.2
15	42	— 7.7 ± 0.9	6.1
20	38	— 10.6 ± 0.7	4.1

corded in the same experiment. This calculation was made from the pulse-oxygen consumption diagram derived from the morning series on the bicycle ergometer carried out during the same period as these forest experiments. The diagrams were calculated in the ordinary way with the help of the method of least squares. For theoretical reasons the oxygen consumption per minute had to be used as a dependant variable in this case. Yet in practice about the same oxygen consumption in the forest was obtained from the diagrams whether these were calculated in the manner described or whether the pulse-rate was used as a dependant variable. This agreement is explained by the very high correlation between the pulse-rate and the oxygen consumption in the bicycle ergometer series.

For subject B. N. there were six such morning series from 6/9 to 8/10, 1945, which gave the mean equation:

$$(\text{O}_2 \text{ in litres per minute}) = 0.0327 (\text{pulse/min.}) - 1.685.$$

For the subject T. L. who did not belong to the group which was more regularly controlled with regard to physical capacity only one series of ergometer experiments was done, and this gave the result:

$$(\text{O}_2 \text{ in litres per minute}) = 0.0293 (\text{pulse/min.}) - 1.837.$$

Results

These are given in Table 34. It will be seen that in 13 of the 16 groups a good agreement within ± 10 per cent was obtained between the oxygen consumption actually measured and that computed from the working pulse-rate for the same experiment. The agreement is remarkably good when one considers the very small series of experiments and takes into account the fact that the forest experiments were done on a series of days and were distributed over the whole day. It should be mentioned further that the pulse counts in the forest were made by a relatively untrained forest ranger. Without doubt an even better agreement could have been reached if the bicycle ergometer calibrations had been done on the same day as the pulse counts during the practical work in question, and if more experiments had been made.

For all the experiments taken together the mean of the differences between the calculated oxygen consumptions and the consumptions actually measured was -0.061 ± 0.021 litres. This mean differs significantly from zero and thus a certain degree of systematic error is to be reckoned with. But this is so small that it may be neglected, being only about a few percent of the real oxygen consumption during heavy industrial work.

The standard deviation in this case was 0.29 litres. χ^2 -analysis (cf. BONNIER and TEDIN 1940) indicates that the readings can be looked upon as normally distributed ($\chi^2 = 11.60$, 13 degrees of freedom, $0.7 > P > 0.5$). Thus the standard deviation may be used to calculate the number of pulse counts (n) necessary to reach a certain accuracy when using the pulse-rate method to determine the oxygen consumption during work in the forest. If the accuracy is required to be 0.1 litres with a probability of 99.7 % (3 times the standard error) n is derived from

$$\frac{3 \cdot 0.29}{\sqrt{n}} = 0.1.$$

In this case n is seen to be 74.

In conclusion it may be said that the working pulse-rate computed from the time for the first 10 beats after cessation of work is

TABLE 34. *Deviations of the oxygen consumption rate calculated on the basis of the working pulse-rate, from the consumption actually measured. Firewood-cutting experiments.*

Subject	Type of work	Number of exp.	Pulse count mean	Measured O_2 l/min. mean	Calculated O_2 l/min.	Deviation %
B.N.	Tree-felling	10	106.6	1.69	1.82	+ 7.7
	Trimming of felled trees	23	99.1	1.68	1.56	- 7.1
	Cross-cutting	11	94.7	1.11	1.42	+ 0.7
	Cleaving of soft-wood	15	107.5	1.89	1.83	- 3.2
	Cleaving of birch-firewood	10	104.6	1.76	1.74	- 1.1
	Barking in strips ..	10	89.0	1.07	1.22	+ 14.0
	Stacking of fire-wood	10	93.7	1.18	1.38	+ 16.9
	Dragging of fire-wood	15	101.0	1.80	1.62	- 10.0
	Tree-felling	9	137.5	2.28	2.20	- 3.5
T.L.	Trimming of felled trees	19	120.3	1.78	1.68	- 5.6
	Cross-cutting	9	122.5	1.79	1.75	- 2.2
	Cleaving of soft-wood	10	128.4	2.07	1.92	- 7.2
	Cleaving of birch-firewood	10	128.7	1.87	1.94	+ 3.7
	Barking in strips ..	10	106.9	1.35	1.29	- 4.4
	Stacking of fire-wood	10	110.9	1.42	1.40	- 1.4
	Dragging of fire-wood	10	127.1	2.24	1.88	- 16.1

quite suitable in practice for the purpose of calculating the metabolic rate during industrial work when very exact determinations are not necessary, and furthermore that the pulse-count in field research may be done by relatively unskilled assistants.

Blood Sugar During Lumber Work

Previous Investigations

In general during heavy work the glucose content of the blood momentarily sinks somewhat when work is commenced (HOHWÜ CHRISTENSEN, 1931). If the exercise is not too prolonged there is an increase above the resting level at the end of the work. This rise may occur before the end of the work, an effect which HOHWÜ CHRISTENSEN ascribes to psychic factors. STRANDELL (1934) maintains that the rise after work may be caused by protracted adrenaline action. In protracted heavy work HOHWÜ CHRISTENSEN found a gradual sinking of the blood sugar towards 0.07 per cent. This low level continued for a certain period after cessation of work and was regarded as a sign of depleted carbohydrate stores in the body. Reduction of blood sugar in protracted heavy work has also been observed by LEVINE, GORDON and DERICK (1924), GORDON, KOHN, LEVINE, MATTON DE M. SCRIVER and WHITING (1925), BEST and PARTRIDGE (1929), KNOLL and LÜSS (1934), DILL, EDWARDS and DE MEIO (1935) and by GOVAERTS and DELANNE (1939).

HOHWÜ CHRISTENSEN and HANSEN (1939) found that the blood sugar content during continued heavy work may fall to values below 0.05 per cent coinciding with a gradual lowering of the respiratory quotient, and that there is an obvious correlation in time between this pronounced fall of blood sugar and the onset of fatigue and other discomforts which compel the man to stop work. When the subjects were given carbohydrate in readily absorbed forms their working capacity was restored, as well as their feeling of well-being. At the same time the blood sugar level rose whilst, however, the respiratory quotient remained at a low level, thus indicating a continued excessive utilisation of fat in the working

muscles. The authors interpret the findings to mean that the onset of fatigue symptoms at low blood sugar levels is not caused by any demand of the muscles for a certain rate of carbohydrate combustion but by the fact that the central nervous system requires a certain blood sugar level to maintain its function. The favourable effect of extra supply of carbohydrates during the course of prolonged heavy work was later demonstrated by DILL, EDWARDS and TALBOTT (1932), HAGGARD and GREENBERG (1941) and by HELLEBRANDT and KARPOVICH (1941). On the other hand experiments by HOHWÜ CHRISTENSEN and HANSEN (1939) show that single larger quantities of carbohydrate supplied immediately before the beginning of the work may give the paradoxical effect that the blood sugar during work falls to very low values. They explain this by assuming that there is a summation of two factors which both operate to deprive the blood of glucose, namely, the glucose storing mechanisms mobilized by the large carbohydrate intake on one hand and the muscular work on the other.

In addition to the influence of nutrition factors and the load and length of work there may also conceivably be a direct effect upon the blood sugar value associated with the time of day. MÖLLERSTRÖM and ULLMARK (1940) state, however, that this does not exert any special influence.

Previous work by LUNDGREN and ZOTTERMAN (1943) has shown that the blood sugar during the course of the working day in lumber work may reach such low levels as to be, in all probability, a direct cause of fatigue. Their experiments also showed that this fall in blood sugar could be avoided by the repeated supply of small amounts of sugar, and this was also accompanied by a subjective improvement. In only one subject were these effects seen, however, and accordingly in the following work an attempt has been made to enlarge the experimental basis.

Methods

Blood sampling and blood sugar analysis were done according to HAGEDORN's and JENSEN's method (1918, 1923) following the in-

structions of JORPES and THANING (1939). Blood samples were taken immediately after stopping work as it was practically impossible to collect blood during continuous lumber work. Thus the real working values were not obtained, because according to HOHWÜ CHRISTENSEN (1931), a pause of even less than one minute causes a change in the blood sugar level. In the pause which follows work of relatively short duration there is a rise in blood sugar above the working level. But in the pause following prolonged heavy work which depletes the carbohydrate stores, the fall in blood sugar persists. Because of these findings of HOHWÜ CHRISTENSEN the values given here can certainly not be looked upon as true working values, but if on the other hand a fall in blood sugar occurred after a long period of work it is improbable that it would disappear before the blood sample could be taken. To control the method, 76 double determinations were made. This gave an error of method = ± 3.7 mg %.

The present experiments comprise examinations of eight subjects during the course of normal working days with ordinary meals, and on these only single samples were taken. In addition to these, two men were subjected to more detailed examination in which the order of meals was varied. On the latter two subjects double samples were taken throughout.

Results

In addition to fasting tests, seven to eight blood samples were collected from the eight subjects who were examined during normal working days with normal meal times during the work. The tests were distributed over the whole working day in such a way that they were sometimes taken immediately after the meals and sometimes after the longest continuous working periods. Because single samples were taken, it is of less interest to give an account of the individual values here. It may be mentioned briefly, however, that in no case were any exceptionally low blood sugar values observed, the lowest value being 75 mg %.

The two men who were examined with double tests were first

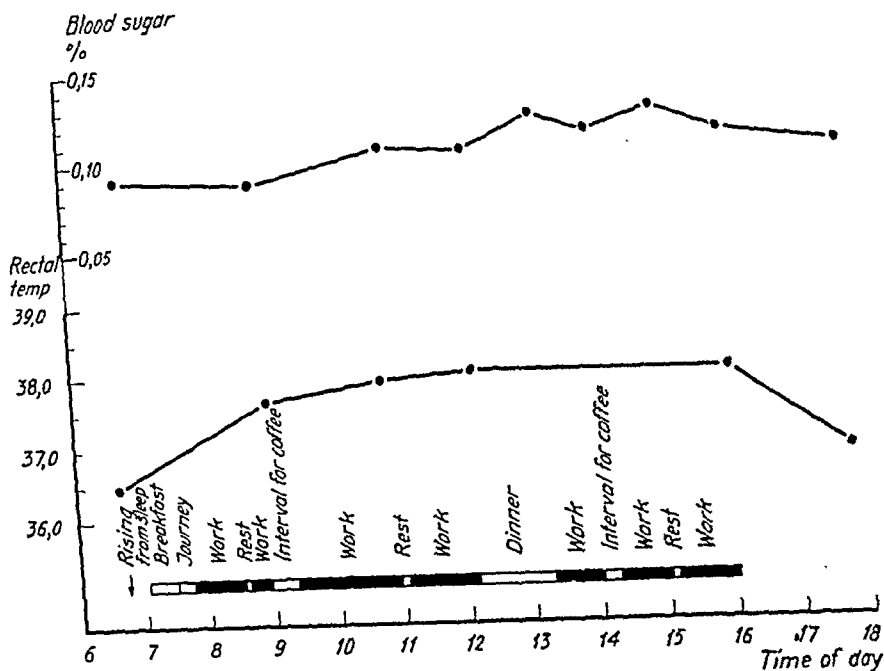


Fig. 29. Blood sugar and rectal temperature during a working day in the forest. Ordinary meal times. Subject G.H.

submitted to a series of tests during normal working days with ordinary meal hours. To control the working intensity the rectal temperature was repeatedly measured. Fig. 29 illustrates the experiments for such a day. It shows that the blood sugar has remained well up during the total working time and that the rectal temperatures indicate a uniform intensity of work. Similar results were obtained from other work days of this kind.

As no fall in blood sugar thus appeared during normal work days with ordinary meal times it was of interest to examine the conditions during long continued work without meals. These experiments were made in such a way that the men had their breakfast in the normal way after which they worked in the forest as long as they could without eating.

The total working time until exhaustion for the subject G. H. varied from 5.90 to 6.52 hours with an actual working time of from 5.18 to 5.75 hours. The corresponding values for E. L. were 6.40

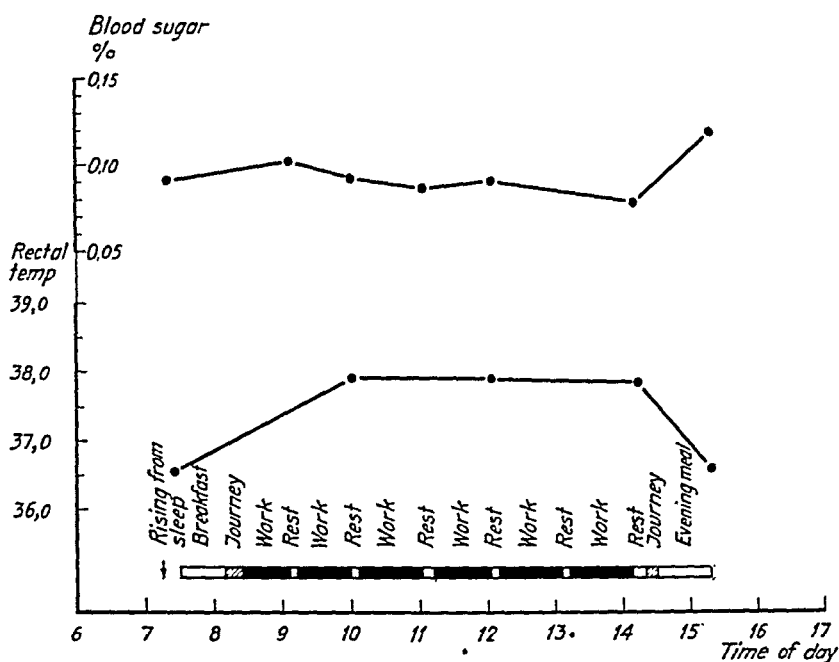


Fig. 30. Blood sugar and rectal temperature during a working day in the forest. No meals during the working time. Subject G.H. The man felt weak during the last few hours of work.

to 6.60 and 5.42 to 5.72 hours. During the working hours the rectal temperatures were found always to lie completely within the normal working range of the subjects, and was for E. L. about 38.0° C, and for G. H. about 37.9° C. The latter subject, G. H., showed a fall of about 0.1° C in records made at the end of the working day. Fig. 30 represents such an experiment and it will be seen that the blood sugar level has a moderate falling tendency but that there are no explicitly low values. All other experiments led to similar results. Values lower than 73 mg % were never obtained. Subjectively, the men reported an increasing dullness and growing fatigue during the last two hours of work.

No experiments were done in which the men went to work without any morning meal whatever, for the sufficient reason that lumber work in practice is never done under such conditions.

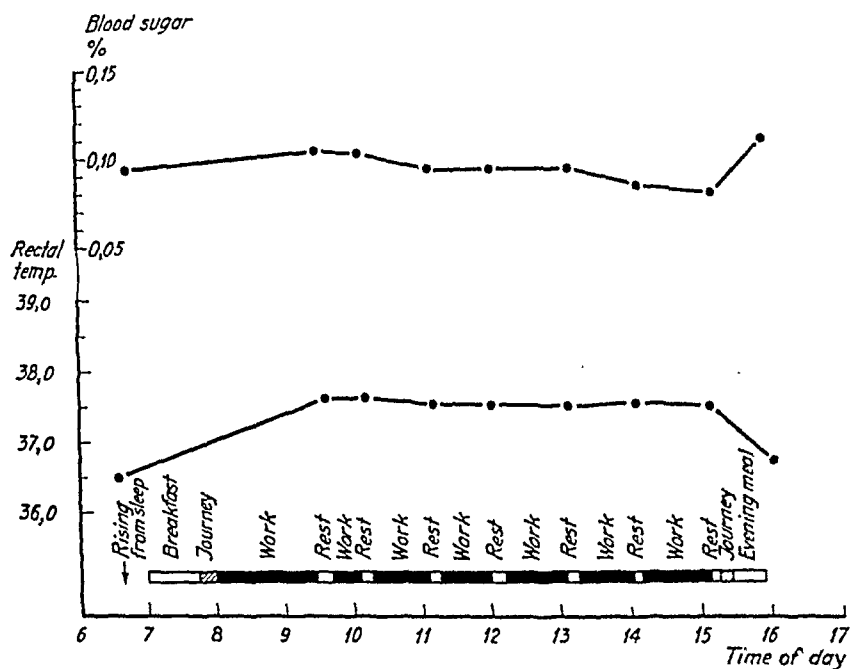


Fig. 31. Blood sugar and rectal temperature during work in the forest after 200 grams of cane sugar had been taken at breakfast. No meals during working time. Subject G. H. The man felt sick at the commencement of work, and felt weak during the last two hours.

HÖHWÜ CHRISTENSEN and HANSEN (1939) demonstrated that the intake of large amounts of carbohydrates before work may cause a pronounced fall of blood sugar during work, and accordingly it was of interest to inquire whether this is of importance in lumber work. The procedure was the following: the man took an extra amount of ordinary beet or cane sugar at breakfast before work whereupon he worked in the forest without meals until exhaustion. The amount of sugar first prescribed was 200 g but the subjects could not tolerate this. They felt ill and E. L. even vomited at the beginning of work. For these reasons 150 g of sugar were taken but even this amount caused disturbance during the first working hour although no vomiting.

An experiment of this kind is represented in Fig. 31. It will be seen that even after this intake of extra sugar the blood sugar has a

slight tendency to fall during the course of the working time although there were no exceptionally low values. Similar results were also obtained for the subject E. L. As is shown in Fig. 31 the rectal temperature level of G. H. was lower than in previous experiments thus showing that the intensity of work was lower here. E. L., however, reached his usual working temperature of about 38° C.

The working periods until exhaustion in these experiments were, for G. H., from 6.20 to 7.25 hours with an actual working time of 5.37 to 6.10 hours. For E. L. it was possible to make observations with extra sugar supply on one day only without vomiting. The corresponding times until exhaustion for him were 6.97 and 6.05 hours. Thus it appears that these times are of the same order of magnitude as those given previously for work days without meals and without extra sugar intake during the work. Subjectively the men experienced some indisposition at the beginning of work and a growing dullness during the last two hours of work.

In these experiments with extra sugar intake before work a pronounced fall of blood sugar may have occurred coinciding with the feeling of sickness during the first hour of work. No blood tests were made during this hour, however. At all events there were no symptoms of fatigue sufficient to cause cessation of work.

To sum up, the result emerges that lumber work in general is not of such intensity as to produce a pronounced fall of blood sugar even during very long periods of work without meals. Intake of unusually large amounts of sugar before commencement of work is unfavourable, as it leads to discomfort during the first part of the working period.

The low blood sugar values found in a previous investigation (LUNDGREN and ZOTTERMAN 1943) might depend upon a special lability of blood sugar regulation of the subject in question or upon an inferior state of physical training which made him work in a more carbohydrate wasting manner (cf HOHWÜ CHRISTENSEN and HANSEN 1939).

Summary

The investigation deals with the following questions:

- 1) The physiological effects of time schedule work on lumber workers.
- 2) The value of some tests on the function of the oxygen transport apparatus.
- 3) The influence of lumber work and other factors on the relation between pulse-rate and oxygen consumption during work.
- 4) The applicability of the body temperature and the pulse-rate as measures of the metabolic rate during heavy industrial work.
- 5) The blood sugar content during lumber work.

1. The physiological effects of time schedule work on lumber workers

- a) The investigation was done on five trained lumber workers of ages ranging from 26 to 55 years, who worked in the forest on a fixed time schedule of working hours during a period of from 9 to 14½ months.
- b) Continuous examinations of the general state of health showed that the lumber work during this period did not produce any signs of overstrain or any other unfavourable bodily symptoms.
- c) An unchanged or somewhat improved adaptation to work of the oxygen transport apparatus was indicated by daily determinations of resting pulse-rate, resting arterial blood pressure, and Schneider index as well as by submaximal work tests on the bicycle ergometer performed about once a week.

2. The value of some tests on the function of the oxygen transport apparatus

- a) The pulse-rate in the morning, before rising from bed and taking of food showed a systematic trend downwards during the period of observation for all the experimental persons. In the light of previous literature these changes may be interpreted as an expression of a continuous improvement in the state of physical training. The fluctuations of blood pressure in the morning in a recumbent position, fasting, did not show any clear systematic changes. The Schneider index showed an improvement for some of the subjects and remained unchanged for the rest.

The pulse-rate showed a greater sensitivity than the other resting tests to "chills", consumption of spirits or strenuous occupations in the previous evenings associated with insufficient sleep. Contrary to the effects of these, there did not occur any significant deviations from the normal level on mornings following upon athletic exercises during the previous evening.

- b) The work tests on the bicycle ergometer were performed in the morning before food intake and consisted of a submaximal series of work experiments at four different loads during which the pulse-rate and the respiratory gaseous exchange were determined for each load. On the basis of these four experiments a pulse- O_2 -diagram was plotted for each experimental day. The interpretation of the test as an expression for the physical capacity was done: (1.) from the pulse-rate level of this diagram at a constant rate of oxygen consumption, (2.) from the slope of this pulse- O_2 -ratio and, (3.) from the average ventilation of the lungs per litre oxygen consumed for all four experiments.

For each one of the factors employed as physical capacity tests the standard error of the single experimental day as well as the deviations between different experimental days were calculated, the systematic trends due to changed state of training being eliminated as far as possible.

- c) Statistical correlations were calculated between the different factors which were used as tests for expressing the physical capacity. This was done for the changes from day by day as well as for their long time trends.

3. *The influence of lumber work and other factors upon the relation between pulse-rate and oxygen consumption during work*

- a) Day-long lumber work may cause a rise of the working pulse-rate at a given rate of oxygen consumption in arm work as well as in leg work.
- b) This rise of the working pulse-rate is caused at least partly by hydrostatic factors associated with blood deposition in the legs.
- c) The rise of the working pulse-rate and the effect of hydrostatic factors upon it are dependent upon the quantity of work performed during the day and it is accentuated by "chills" and other factors deteriorating the physical fitness.

4. *The use of the body temperature and the pulse-rate as a measure of the metabolic rate during heavy work*

- a) For most subjects the rectal temperature was higher in the afternoon than in the morning when working on the bicycle ergometer at the same metabolic rate.
- b) At a given metabolic rate the rectal temperature level was of the same order of magnitude in all the lumber workers examined. In an untrained subject, however, it was definitely higher.
- c) The body temperature during work did not show with certainty any seasonal variations.
- d) The rectal temperature during lumber work may well be used as an expression of the mean metabolic rate.
- e) The measurement of the time for the first ten pulse beats

after stopping work on the bicycle ergometer is shown to give a good expression for the pulse-rate during actual work.

- f) It was found that for most practical purposes sufficiently accurate results were obtained by determining the metabolic rate from the pulse-rate during work after previous measurement of the subject's pulse-o₂-ratio in bicycle ergometer experiments.

5. The blood sugar content during lumber work

The blood sugar content during lumber work with the usual order of meals varied within normal limits. Nor did any marked changes appear when the subject worked until exhaustion without any meals. This was true both when the morning meal previous to work was of usual composition and when it contained a great amount of sugar.

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Studies of the Arterial Pulse
Wave, Particularly in the Aorta

By

I. G. PORJÉ

Stockholm 1946

Dedicated to
the memory of Aron Apéria

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Preface.

Owing to the decease of A. APÉRIA, important hemodynamic research was quite suddenly interrupted. In the present study I have taken up some of the numerous hemodynamic problems discussed in APÉRIA's thesis.

To Professor JOSUA TILGREN, who has not only aroused my interest in studies of hemodynamic problems, but has also assisted me in every conceivable way, I desire to convey my most cordial thanks. I am also greatly indebted to Professor G. LILJE-STRAND, who has given me valuable advice and has looked through the present work.

B. ANDERSSON, Licentiate of Technology, who has been my mathematical adviser, has given me invaluable assistance in the production of the present work. I am deeply grateful to him for this friendly cooperation. I am also indebted to N. O. JOHANNES-SON, student of technology, who has assisted me in making a number of the calculations, and with whom I have had the privilege of discussing physical problems.

I have had the great privilege of using the apparatus constructed by Dr. RUNE ELMQVIST. His expert knowledge and helpfulness have been invaluable.

My material has been examined from a statistical point of view by Docent L. GOLDBERG. For his valuable advice and assistance I owe him a debt of gratitude.

I desire here to convey my cordial thanks to Dr. S. NAUCKHOFF and to Nitroglycerin AB., who, in a munificent way, have financially assisted my work.

To my wife, ANN-MARI PORJÉ, who has acted as my assistant, I am deeply grateful for the skill and accuracy with which she has assisted me, as well as for the great interest she has shown in my work.

In conclusion, I desire to acknowledge my indebtedness to AB. Elema, who, for a number of years, have placed an amplifier

device, free of cost, at my disposal, and to Mr. AHLSTRÖM in the same firm, who has assisted me in testing the apparatus. The expenses have been defrayed by grants from "Karl Petrén's minnesfond", "Nordisk Insulinfond" and "Svenska Sällskapet för Medicinsk Forskning".

Since the Spring Term of 1946 I have had the great privilege of holding a state scholarship for the furtherance of higher medical studies at the Caroline Institute.

Stockholm, september 1946.

I. G. Porjé.

Introduction.

This work constitutes a study of the nature of the pulse wave under certain physiological conditions and in cases of circulatory disturbances. In his work "Hemodynamical Studies" (1940), APÉRIA has shown theoretically and experimentally that the formulation which hemodynamics has received, with the so-called air chamber theory as its leading principle, has been based upon a number of erroneous premises. Therefore its practical application has given misleading results in, for example, the determination of pulse wave velocity and of magnitudes calculated with the aid of that velocity, such as the elasticity modulus, the stroke volume of the heart, and peripheral resistance. According to APÉRIA, the undulatory theory, based on the equations given by L. EULER in 1775, ought to be the leading principle for hemodynamics.

In this work the undulatory theory has been applied in the study of pressure oscillations in the aorta. Pulse curves have been recorded simultaneously from the left subclavian artery and the left femoral artery, and in some cases from the abdominal aorta also.

The great obstacle in the study of the pulse wave has been the lack of a "central" theory including the reflection relations of the wave. Up to the present this lack has stood in the way of an exact treatment of hemodynamics. APÉRIA expresses the situation in the following way: "The absence of an exacter theory of reflections is for the present the greatest obstacle in the way of a quantitative theory of circulation, which will be more satisfactory for practical purposes. Further real advances of the theory are to be looked for in this direction. An improvement could first be made by the introduction of suitable characteristic constants and functions on strictly hydrodynamical foundations." It is possible, however, to give a picture of the reflection mechanism of the pulse wave with the aid of the undulatory theory.

The work is in three parts. In the first is given a short summary of the earlier works which deal with the theories applied. Since my own views differ in many respects from those currently and previously accepted, I have not included in this work an actual historical survey of hemodynamics. Those interested are referred to APÉRIA's dissertation, mentioned above. In the second part, an account is given of the methods used, the apparatus, and the physical-mathematical mode of procedure. The third part consists of experimental studies and the results thereof.

arterial pulse". The aorta is regarded as a manometer which records the central pulse (art. subclavia) in the form of the femoral artery (art. iliaca) pulse. The fundamental oscillation of the arterial pulse is thus the self-oscillation of this manometer. The self-oscillation time and damping in the manometer can then be calculated with the aid of Fourier-analysis of the central and peripheral pulse curve. B. ANDERSSON and I. G. PORJÉ have subjected this work of BROEMSER's to a critical examination with special attention to the basic equations. They found that BROEMSER's assumption regarding the mechanics of the manometer was oversimplified, but that the self-oscillation time could be calculated for 18 healthy subjects.

MATOKA and KAJIURA (1933) have studied the radial pulse curves of 10 normal and 10 sclerotic subjects with the help of harmonic analysis. The pulse period is divided into 24 intervals and analyzed. These authors found that the amplitudes of the higher frequencies are higher in the sclerotic pulses. The mathematical analysis is unsatisfactory, however, since this division into intervals is not sufficient to make possible the calculation of the amplitudes of the higher frequencies. (Examination of the mathematics carried out by B. ANDERSSON.)

About 100 years ago E. H. WEBER made the first experimental examinations of the pulse wave. He also carried out thorough model experiments with caoutchouc tubes and the small intestine. E. H. WEBER is probably the first who tried to determine the velocity of the pulse wave in man. By measuring the difference in time between the stroke of the pulse wave in the facial artery and the anterior tibial artery, this velocity could be estimated in one experiment at 9,240 mm. per second (the velocity = the path of the pulse wave divided by the difference in time).

In the year 1866 W. WEBER was able to give the formula for the speed of propagation (a) of waves in elastic tubes filled with incompressible liquid. $a^2 = \frac{r}{2a\sigma}$, where r = the radius of the tube,

σ = the specific density of the liquid, a = the differential quotient, $\frac{dr}{dp}$ and p = the pressure. v. KRIES (1892) points out that

the value $\frac{dr}{dp}$ depends not only on the qualities of the wall but also on the radius of the tube. v. KRIES writes the formula

$\alpha = \sqrt{\frac{\Delta p}{\Delta Q} \cdot \frac{Q}{\sigma}}$, in which Q = the cross-section of the tube, σ = the specific density of the liquid, and Δp = the increase in pressure which produces an enlargement of the cross-section with the value ΔQ . $\frac{\Delta p}{\Delta Q}$ also depends not only on the qualities of the wall

but also on the radius of the tube. $\frac{\Delta p}{\Delta Q} \cdot Q = \frac{\delta \cdot E}{2r}$, in which δ = the thickness of the wall and E its elasticity modulus. The formula is thus practically the same as that given by MOEN (1878), namely: $\alpha = 0.9 \cdot \sqrt{\frac{\delta \cdot E \cdot g}{2r \cdot \sigma}}$, in which g = the acceleration of the force of gravity, E expressed in grams per cm².

O. FRANK, who criticizes the formulae mentioned above because they do not take into account the longitudinal stretching of the vessel, evolved a formula which does take account of this relation. The formula is as follows: $\alpha = \sqrt{\frac{\kappa}{\sigma}}$; $\kappa = \frac{\Delta p}{\Delta V} \cdot V$; Δp = the increase in pressure and ΔV the corresponding increase in volume of the volume of the vessel V . In this work V is considered to constitute a small volume element. The formula is even extended to apply to the whole so-called air-chamber. κ FRANK calls the volume elasticity modulus. Theoretically this formula is different from those given by MOEN and v. KRIES in that the "volume elasticity modulus" in itself includes both the transverse and longitudinal modulus of the vessel, because

$$\frac{\Delta V}{\Delta p \cdot V} = \frac{r}{\delta} \cdot \left(2 \cdot \frac{1}{E_q} + \frac{1}{2 E_L} \right)$$

E_q = the transverse modulus of the tube; E_L = the longitudinal modulus of the tube and then $\frac{1}{E} = \frac{1}{E_q} + \frac{1}{4 E_L}$; $E = \frac{E_q \cdot 4 E_L}{E + 4 E_L} =$

the combined modulus. $\therefore \alpha = \sqrt{\frac{\delta \cdot E}{2r \cdot \sigma}}$

ALOYS MÜLLER gives a formula for the velocity of the pulse wave which holds even for vessels with thick walls. According to this author the square of the pulse wave velocity is proportional to the elasticity modulus of the wall of the vessel multiplied by the relation $\frac{\text{area of cross-section of vessel wall}}{\text{area of entire cross-section of vessel}}$.

The formulae above hold good under the conditions mentioned for an advancing wave. — When the pulse wave is not an advancing wave, so-called corresponding points have been sought on simultaneously recorded central and peripheral pulse curves. The reflections are supposed to be least at these points, which have usually been located at the base points of the curves. Since, however, the bases are often not in the form of points, FRANK locates the corresponding points at $1/5$ of the heights of the ascending limbs. BROEMSER and RANKE regard the so-called first turning-points of the ascending limbs as corresponding. The velocity of the pulse wave then is like the relation:

$$\frac{\text{distance between measuring pts.}}{\text{difference in time between the corr. pts.}}$$

O. FRANK, who emphasizes the significance of reflections, nevertheless does not take them into account in his development of the air-chamber theory. WEZLER and BÖGER compare the so-called air-chamber to a pipe, closed at one end. A standing wave is formed of reflections at the aorta valves and at a distance of $\frac{\text{length of the wave}}{4}$ from that place. The oscillation time of the

wave is determined by the difference in time between two maxima on a pulse curve from the femoral artery. This analogy is extraordinarily simplified and the calculation of the oscillation time is physically unfounded. Such a standing wave would be rejected under Fourier-analysis; nor has it been possible to prove the existence of such a wave with the oscillation time given by WEZLER and BÖGER (0.27—0.50 seconds). In his work "Hemodynamical Studies" (1940), APÉRIA stresses the great uncertainty which attaches to the determination of the velocity of the pulse wave. According to APÉRIA, one has the following to take into consideration. If the pressure is represented by $P(x, t)$, then according to d'ALEMBERT the wave can be written $P(x, t) = f(x - ct) + g(x + ct)$, in which f and g are arbitrary functions of the special arguments $(x - ct)$ and $(x + ct)$, c = the velocity of the wave, and t = the time. The pulse wave P can be regarded as the sum of the waves f and g . It is, for example, possible to call forth directly by physical means an advancing wave φ with a constant velocity c , which is reflected with the same velocity c , although often with dampings and deformations. Through a new reflection the reflected wave ψ can even come into the original wave, etc. All waves which move in the positive direc-

tion unite with a single wave, f ; those which move in the negative direction, with a single wave, g . These facts can be applied in hemodynamics. In the determination of pulse wave velocity, one assumes that one may take g as approximately equal to 0, whereupon it remains only to take the advancing wave f in to consideration. c is then $= \frac{x}{t}$; for a strictly advancing wave

t is the time difference between corresponding points (points situated on the same level) on two pressure curves lying near one another, and x is the distance between the places where the curves are traced. If a retrograde wave is added to an advancing wave $f(x - ct)$, the measuring becomes difficult to carry out. Moreover, it is impossible at present to record pulse curves lying near one another on, for example, the aorta or subclavia. Since deformation of curves lying near one another has already come into existence, it is still more difficult to find points on curves from the subclavian artery and femoral artery on the same pressure level which correspond. APÉRIA thinks that measuring the time difference between the minimal points of the curves should give the best results. When these do not appear it is suggested that one measure $1/4$ or $1/5$ of the height of the ascending limbs of the pulse curves.

HAMILTON and DOW (1939) have studied, with the aid of a special manometer, a series of pressure curves on dogs from the aortic arch down to the femoral artery. They found that pulse pressure increases gradually from the aortic arch to the femoral artery, although the mean pressure remains constant. Changes in form and pressure are ascribed primarily to reflections and changes in the volume elasticity modulus of the vessel. Standing waves arise which fuse with the fundamental form of the pulse. They believe that they have also found that the velocity of the pulse wave increases considerably on its way down from the heart to the femoral artery. They assume, however, without giving any physical explanation, that the base points of the curves correspond.

Thus, in spite of the many works which treat hemodynamical questions from the physical, physiological, and clinical points of view, there are many concepts which are obscure and physically inexact. The so-called velocity of the pulse wave, the establishment of which is to some extent the key to the calculation of the majority of hemodynamic magnitudes, has been determined with

the physically unfounded assumption that at certain points on the pulse curves the wave is advancing. *It has thus been assumed that a certain portion of the wave front advances without deformation. But this is not true, and therefore the conclusions based on the assumption are misleading.* Up to this time, the reflection relations of the pulse wave have been accorded extraordinarily little study. As yet no theory has been worked out which includes the reflection of the wave, and this has prevented a more exact development of hemodynamics.

Therefore the author has studied some of the basic problems of hemodynamics. They may be summarized thus:

1. Study of the pulse wave under certain physiological and pathological conditions with Fourier-analysis, by means of which the course of oscillation is divided up into simpler sine oscillations.

2. Establishment of certain laws for the reflection of the pulse wave under these conditions. Knowing these laws, it was possible to determine the velocity, important from the physiological and clinical points of view, of an advancing wave in the aorta in healthy subjects and in certain cases of circulatory disturbances. Some fundamental information about certain qualities of the vessel wall and dynamic conditions in the vessels has been obtained. The possibility of studying reflections has opened new perspectives for further studies and understanding of hemodynamic problems under physiological and pathological conditions. The experimental foundation consists of pulse curves recorded simultaneously from the subclavian artery and femoral artery (art. iliaca) and in certain cases from the subclavian artery and the abdominal aorta.

2. The measuring instrument must not cause changes in the phenomenon to be recorded.

3. A suitable amplifying device and suitable connections must be employed. An alternating current amplifier with relatively long time constant is especially suitable for this purpose. By such means it is possible to record correctly the relatively low frequencies which one has to do with up the whole scale to and including the very high frequencies.

4. It must be possible to use the apparatus in the clinic without inconvenience and without any loss of accuracy.

The qualities of the apparatus have been tested by the designer, Dr. RUNE ELMQVIST, and also by Docent PER BRÜEL of Chalmers Technical Institute. They found that frequencies from 0.5 to 100 c/s could be given without amplitude distortion and that the amount of the reading was directly proportional to the variation in pressure. Thus the apparatus is well suited to the recording to the arterial pulse. Comparison with curves recorded under the same conditions with the condenser microphone and a special amplifier show almost the identical course. (The apparatus was kindly put at my disposal by Dr. TYBJÆRG HANSEN, Rigshospitalet, Copenhagen.)

Point 2 has been tested by pressing the microphone down to different depths in the tissues (within reasonable limits). In this way one obtains either no change at all in the curves or possibly an increase or decrease in the reading, but no deformations.

Mathematical-Physical Methods.

In the pulse curves which form the basis of these studies, the ordinate indicates the variation in pulse pressure and the abscissa, the time. The variations in pressure can then be regarded as a periodic function of the time. The time period = the pulse time. Deviations from the periodicity are very slight. Periodic movements of the type which appear in mathematical-physical problems, such as pendulous motions, under which heading we may include wave movements, are often studied with the aid of Fourier-analysis, partly because the equations which underlie these phenomena lend themselves to such treatment, and partly because Fourier-analysis is a particularly well-developed mathematical

method. Vibration equations are simplified to such a degree that they become linear differential equations.

The simple, universally-known example of such a wave-movement is a vibrating string. If the displacement of the string at a certain point is studied, as a function of the time, one finds that this displacement is a periodic function which can be analyzed by Fourier-analysis into simpler vibrations. (By the use of appropriate devices the string can be made to vibrate with only one of any of the time periods of these vibrations.) The different vibrations can occur independently of one another.

We may take an example from acoustics. Every sound, regardless of the way in which it arises, can be analyzed with the aid of Fourier-analysis into simple sine vibrations, fundamental tone and overtones. In a given medium, such as air, the different tones are transmitted with the same velocity. This velocity depends on certain qualities of the medium, above all its specific weight and its elasticity. The different tones can, however, be reflected differently if, for example, they strike a wall.

The above can, under certain conditions, be applied to the pulse wave. If a central pulse curve (subclavian artery) and a peripheral pulse curve (femoral artery) are recorded, important information about the nature and transmission of the pulse wave can be obtained with the help of Fourier-analysis.

The vibration equations for the pulse wave can in the first approximation be shown to be linear (EULER, W. WEBER, KRIES, AFÉRIA). The mathematical-physical foundation for this assumption is not included in this work. Those interested are referred to the works of the authors mentioned. The physiological-physical foundation is that the variations in arterial diameter are small during the pulse stroke, while large amounts of liquid are streaming through the vessel. The elasticity modulus for every little cross-section is then to be regarded as a constant when subject to pressure vibrations around a definite mean pressure. The radial velocities and accelerations in the blood become small and destructible in comparison with the axial ones. Thus one needs to take into account only the axial velocities and accelerations, and furthermore the pressure over every cross-section remains constant.

Just as a sound can be analyzed with the aid of Fourier-analysis into a fundamental tone and overtones, the pulse wave also can be analyzed into simple sine vibrations, each of which can be examined by itself, independently of the others. The vibrations

are considered to take place around a mean pressure which is also separately treated. If a pulse curve is recorded, one may determine with great accuracy the different sine vibrations of which it is composed. If a central curve and a peripheral curve are recorded simultaneously, one may also study more closely the transmission of every separate sine vibration.

The mathematical procedure in this work has been as follows:

Suppose that the function $f(t)$ is a periodic function of the variable t and that the period is T , that is to say

$$1) \quad f(t + T) = f(t)$$

Under very general conditions the function can be expressed in the form of a trigonometric series

$$2) \quad f(t) = \frac{1}{2} a_0 + \sum_{n=1}^{\infty} \left(a_n \cos \frac{2\pi n}{T} t + b_n \sin \frac{2\pi n}{T} t \right)$$

where a_n and b_n are constants determined by the integrals

$$a_n = \frac{2}{T} \int_0^T f(t) \cos \frac{2\pi n}{T} t dt \quad (n = 0, 1, 2, \dots)$$

3)

$$b_n = \frac{2}{T} \int_0^T f(t) \sin \frac{2\pi n}{T} t dt \quad (n = 0, 1, 2, \dots)$$

The series can also be written

$$4) \quad f(t) = \frac{1}{2} A_0 + \sum_{n=1}^{\infty} A_n \cos n \left(\frac{2\pi n}{T} t - \alpha_n \right)$$

where the amplitudes A_n are determined by a_n and b_n

$$A_n = \sqrt{a_n^2 + b_n^2}$$

and the phase angles α_n are single valued, excepting for multiples of $\frac{2\pi}{n}$, determined by the relation. This equation expresses the fact that every periodic function may always be resolved into a number of simple sine vibrations corresponding to the fundamental and its partials.

$$5) \quad \cos n \cdot \alpha_n = \frac{a_n}{A_n}; \quad \sin n \cdot \alpha_n = \frac{b_n}{A_n}$$

Under very general conditions, furthermore,

$$6) \quad \int_0^T f(t)^2 dt = T \left\{ \frac{1}{4} A_0^2 + \frac{1}{2} \sum_{n=1}^{\infty} A_n^2 \right\}$$

or, in other words,

$$7) \sum_{n=1}^{\infty} A_n^2 = \frac{2}{T} \int_0^T f(t)^2 dt - \frac{1}{2} A_0^2$$

The subsequent periodically variable component of the pressure [p (t)] is of particular interest in the study of pulse curves. The abscissa axis is therefore inserted so that the mean time value of the component of pressure is zero. The function thus assumes the form

$$8) p(t) = \sum_{n=1}^{\infty} A_n \cos n \left(\frac{2\pi}{T} t - \alpha_n \right); \quad T = \text{the pulse time}$$

t = the coordinate of time

From the relation 7)

$$\sum_{n=1}^{\infty} A_n^2 = \frac{2}{T} \int_0^T p(t)^2 dt$$

The pulse curve embodying an undefined ordinate scale is standardized by multiplication with the constant $\left[\frac{2}{T} \int_0^T p(t)^2 dt \right]^{-1}$

The *normal function of pressure* will thus be

$$9) p_n(t) = \frac{p(t)}{\sqrt{\frac{2}{T} \int_0^T p(t)^2 dt}} = \sum_{n=1}^{\infty} c_n \cos n \left(\frac{2\pi}{T} t - \alpha_n \right)$$

where the amplitudes c_n will be

$$10) c_n = \frac{A_n}{\sqrt{\sum_{n=1}^{\infty} A_n^2}}$$

Since

$$11) \sum_{n=1}^{\infty} c_n^2 = 1$$

it will be seen that the magnitudes c_n are suitable values when expressing the parts of the different frequencies in the measured function of pressure.

In determining the constants A_n a harmonic analyzer can be used. For the further determination of c_n it is necessary to know the integral $\int_0^T p(t)^2 dt$. The function $p(t)$ being purely empirically included in a diagram it is necessary to insert also the function of $p(t)^2$. The integral can then be calculated by integrators, or approximately by the aid of some numerical integration method such as the trapezium formula or Simpson's formula. This second stage in the calculation is laborious and many sources of error can arise.

c_n^2 can be determined with adequate accuracy by means of a numerical method as described herewith. A fundamental condition in making use of this method is that the first of the constants c_n^2 dominate, and the function of pressure thus contains no material component the frequency of which is many times greater than the basic frequency $n = 1$.

The function $f(t)$ is an empirically determined function of t with the period T . Suppose that $f(t)$ is known with adequate accuracy, if the value of the function for n equidistant t -values within a period is known. $f(t)$ is taken continually.

The equidistant t -values are

$$t_0, t_1, t_2, \dots, t_n = t_0 + T$$

One can accept $t_0 = 0$.

It is best to select n as a multiple of 4, and in examining the pulse curves n has been taken as 12, so that study will here be directed to this case. The function $f(t)$ assumes for $t = t_n (n=0, 1, \dots, 12)$ the values f_0, f_1, \dots, f_n . It is possible to determine a trigonometrical polynomial of the form

$$12) \quad F(t) = \frac{a_0}{2} + \sum_{m=1}^6 a_m \cos \frac{2\pi m}{T} t + \sum_{m=1}^5 b_m \sin \frac{2\pi m}{T} t$$

which for $t = 0, t_1, \dots, t_{12} = T$ assumes the same values f_0, \dots, f_{12} as the function $f(t)$. The 12 conditions which are applied to the polynomial give 12 equations for determining the 12 constants

$a_0, a_1, \dots, a_6, b_1, \dots, b_5$. As $t_k = k \frac{T}{12}$ these equations become

$$13) \quad \begin{cases} f_k = \frac{a_0}{2} + \sum_{m=1}^6 a_m \cos \frac{2\pi m k}{12} + \sum_{m=1}^5 b_m \sin \frac{2\pi m k}{12} \\ (k = 1, 2, \dots, 12) \end{cases}$$

Knowing that

$$14) \quad \begin{cases} \sum_{k=1}^{12} \cos \frac{2\pi m k}{12} = \begin{cases} 12 & \text{if } m = 0 \\ 0 & \text{if } m \neq 0 \end{cases} \\ \sum_{k=1}^{12} \sin \frac{2\pi m k}{12} = 0 \end{cases}$$

Adding therefore the 12 equations 13) we get

$$15) \quad \sum_{k=1}^{12} f_k = 6 a_0$$

Extended the k^{th} of the equations 13) with $\cos \frac{2\pi\mu k}{12}$, where μ is a whole number, $1 \leq \mu \leq 6$, and rewrite the terms with the aid of the known formulas

$$\begin{cases} \cos \frac{2\pi m k}{12} \cos \frac{2\pi\mu k}{12} = \frac{1}{2} \left\{ \cos \frac{2\pi(m+\mu)k}{12} + \cos \frac{2\pi(m-\mu)k}{12} \right\} \\ \sin \frac{2\pi m k}{12} \cos \frac{2\pi\mu k}{12} = \frac{1}{2} \left\{ \sin \frac{2\pi(m+\mu)k}{12} + \sin \frac{2\pi(m-\mu)k}{12} \right\} \end{cases}$$

Adding the 12 equations we get

$$\begin{aligned} \sum_1^{12} f_k \cos \frac{2\pi\mu k}{12} &= \frac{a_0}{2} \sum_{k=1}^{12} \cos \frac{2\pi\mu k}{12} + \\ &+ \sum_{m=1}^6 \frac{a_m}{2} \left\{ \sum_{k=1}^{12} \cos \frac{2\pi(m+\mu)k}{12} + \sum_{k=1}^{12} \cos \frac{2\pi(m-\mu)k}{12} \right\} \\ &+ \sum_{m=1}^5 \frac{b_m}{2} \left\{ \sum_{k=1}^{12} \sin \frac{2\pi(m+\mu)k}{12} + \sum_{k=1}^{12} \sin \frac{2\pi(m-\mu)k}{12} \right\} \end{aligned}$$

In accordance with formulas 13) all the sums will be zero except $\sum_{k=1}^{12} \cos \frac{2\pi(m-\mu)k}{12}$, which will be zero if $m \neq \mu$ and 12 if $m = \mu$.

It is therefore simple to get

$$6 a_\mu = \sum_{k=1}^{12} f_k \cos \frac{2\pi\mu k}{12}$$

In a similar manner

$$6 b_\mu = \sum_{k=1}^{12} f_k \sin \frac{2\pi\mu k}{12}$$

In these expressions the figures f_k are multiplied with $\frac{\sin \left\{ \frac{2\pi\mu k}{12} \right\}}{\cos \left\{ \frac{2\pi\mu k}{12} \right\}}$ and these factors can only assume the values $0, \pm \frac{1}{2}, \pm \frac{\sqrt{3}}{2}, \pm 1$.

The terms in the sums can therefore be combined in a suitable manner, and computation of a_μ and b_μ can be done in accordance with a diagram as follows:

—	f_1	f_2	f_3	f_4	f_5	f_6
f_{12}	f_{11}	f_{10}	f_9	f_8	f_7	—
s_0	s_1	s_2	s_3	s_4	s_5	s_6
—	d_1	d_2	d_3	d_4	d_5	—
s_0	s_1	s_2	S_3	d_1	d_2	d_3
s_6	s_5	s_4	—	d_5	d_4	—
S_0	S_1	S_2	S_3	σ_1	σ_2	σ_3
D_0	D_1	D_2	—	δ_1	δ_2	—

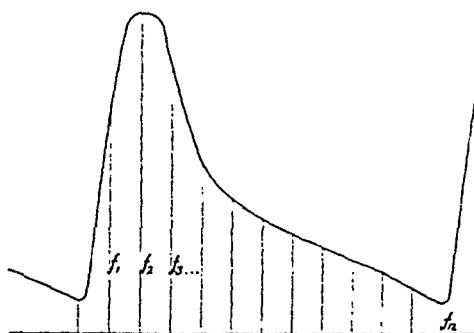


Fig. 1. The pulse time is divided into 12 equal parts and the ordinates f_k are measured.

Cosine								M	Sine							
—	—	D_2	—	$-S_2$	S_1	—	—	$\frac{1}{2}$	σ_1	—	—	—	—	—	—	—
—	—	—	D_1	—	—	—	—	$\frac{\sqrt{3}}{2}$	—	σ_2	δ_1	δ_2	—	—	—	—
S_0	S_1	D_0	—	S_0	$-S_3$	D_0	D_2	1	σ_3	—	—	—	σ_1	σ_3	—	—
S_2	S_3	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
I	II	I	II	I	II	I	II		I	II	I	II	I	II	I	II
$6 a_0$		$6 a_1$		$6 a_2$		—		I+II	$6 b_1$		$6 b_2$		—		—	
$12 a_6$		$6 a_5$		$6 a_4$		$6 a_3$		I-II	$6 b_5$		$6 b_4$		$6 b_3$		—	
a_0	a_1	a_2	a_3	a_4	a_5	a_6			b_1	b_2	b_3	b_4	b_5			

The values f_1, f_2, \dots, f_{12} are inserted as in the diagram (see also figure). The sums $s_0 = f_{12}$, $s_1 = f_1 + f_{11}$, ... are formed together with the differences $d_1 = f_1 - f_{11}$, $d_2 = f_2 - f_{10}$, ... Then the values s_0, \dots, s_6 and d_1, \dots, d_5 are inserted in order, in the same way as in which the figures were given. Afterwards form the sums $S_0 = s_0 + s_6$, ... and $\sigma_1 = d_1 + d_5$, ... respectively, and the differences $D_0 = s_0 - s_6$, ... and $\delta_1 = d_1 - d_5$, ...

The values S_k and D_k are used when calculating the cosine coefficients, values σ_k and δ_k when calculating the sine coefficients, and they should be inserted in the diagram under the respective headings "sine" and "cosine" in the correct places. The sums I and II are then formed by multiplying the figures in a column with the factor included under the heading M on

the same line as the figure, and then adding up by columns. Form the sums I+II and the differences I-II, which in accordance with the diagram represent the Fourier coefficients excepting for one factor. The Fourier coefficients are inserted last.

As an example the calculation of a_4 is appended herewith.

From the diagram

$$\begin{aligned} 6 a_4 &= \text{I} - \text{II} = \left(-\frac{1}{2} S_2 + S_0\right) - \left(\frac{1}{2} S_1 - S_3\right) = \\ &= \left[-\frac{1}{2} (s_2 + s_4) + s_0 + s_6\right] - \left[\frac{1}{2} (s_1 + s_5) - s_3\right] = \\ &= \left[-\frac{1}{2} (f_2 + f_{10} + f_4 + f_8) + f_{12} + f_6\right] - \\ &\quad - \left[\frac{1}{2} (f_1 + f_{11} + f_5 + f_7) - (f_3 + f_9)\right] = \\ &= -\frac{1}{2} f_1 - \frac{1}{2} f_2 + f_3 - \frac{1}{2} f_4 - \frac{1}{2} f_5 + f_6 - \frac{1}{2} f_7 - \frac{1}{2} f_8 + f_9 - \frac{1}{2} f_{10} - \frac{1}{2} f_{11} + f_{12} \\ &= \sum_{k=1}^{12} f_k \cos \frac{2}{3} \pi k \end{aligned}$$

The calculation of c_n^2 is made clear in equations 4 and 10. The phase angles are obtained from equation 5.

Physiological Significance of the Magnitude c_n^2 .

The elasticity modulus for every little cross section is assumed to be constant. The time mean value of the elastic energy for each sine vibration per length of the vessel at the places of measurement is $\frac{Q}{\kappa} \cdot \frac{1}{T} \int_0^T \left[A_n \cos n \left(\frac{2 \pi \cdot t}{T} - a_n \right) \right]^2 dt = \frac{1}{2} \cdot \frac{Q}{\kappa} \cdot A_n^2$.

The corresponding value for the total elastic vibration energy is

$$\frac{1}{2} \cdot \frac{Q}{\kappa} \cdot \sum_{n=1}^{\infty} A_n^2. \quad Q = \text{the cross section surfaces of the vessel; } \kappa = \\ = \text{volume elasticity modulus; } T = \text{pulse time.}$$

Under these circumstances c_1^2 , c_2^2 , c_3^2 , etc., indicate the time mean value of the shares of elastic energy stored in the artery per length of vessel at the place of measurement coming from the fundamental vibration, the first partial and second partial, etc., as is evident from the theory for Fourier series, c_1^2 , c_2^2 , c_3^2 , etc. give a succession of values which characterises the nature of the oscillations in different pulse curves. The oscillations are considered to take place around the mean pressure. See also the equations 7 and 10.

The mathematical error when the approximation method is used amounts to 0.1–4.1 % for c_1^2 , 0.1–3.3 % for c_2^2 and 1.2–14.0 % for c_3^2 . (Calculation of error carried out by Mr. BENGT ANDERSSON.)

The Transmisson of the Pulse Wave.

When earlier investigators (FRANK, BROEMSER, A. MÜLLER, and others) have wished to determine pulse wave velocity, they have used different methods, each of which, generally speaking, gives a different result. The velocity measured thus can scarcely have any physiological significance. One may speak, as has been done before, of corresponding points on two very differently formed pressure curves only by way of definition; from a physical point of view there is nothing which indicates that these corresponding points have anything in common. It must be still more hazardous to draw conclusions with the aid of these velocities about the elastic qualities of the vessel walls, the stroke volume of the heart, peripheral resistance, etc. On the condition mentioned above, that the vibration is approximately governed by linear differential equations, the following alternatives are conceivable:

1. A simple sine-formed pulse wave (possibly slightly damped) moves ahead without being reflected. If the velocity of the wave is known, it is possible, with the aid of the formula

$$v = \sqrt{\frac{\delta \cdot E}{2r \cdot \sigma}} = \sqrt{\frac{x}{\sigma}}$$
 to draw far-ranging conclusions about the elasticity of the vessel wall. This velocity is then independent of the frequency. δ = the thickness of the wall; E = the combined elasticity modulus of the wall; r = the radius of the vessel; σ = the specific density of blood; x = the coordinate of length; t = the coordinate of time.

With $x = x_0$, the pressure is recorded:

$$p(x_0, t) = A(x_0) \cos n \frac{2\pi}{T} \left(t - \frac{x_0}{v} \right)$$

and with $x_1 = x_0 + L$ is recorded simultaneously the pressure

$$p(x_1, t) = A(x_1) \cdot \cos n \frac{2\pi}{T} \left(t - \frac{x_1}{v} \right) = A(x_1) \cos n \left[\frac{2\pi}{T} \left(t - \frac{x_0}{v} \right) - \Delta\alpha_n \right]$$

in which $\Delta\alpha_n$ is an angle which can be calculated directly in connection with the analysis of the pressure curves. From the expression for $p(x_1, t)$ mentioned above one gets:

$$\frac{2\pi}{T} \cdot \frac{x_1}{v} = \frac{2\pi}{T} \cdot \frac{x_0}{v} + \Delta\alpha_n$$

$$v = \frac{2\pi(x_1 - x_0)}{T \cdot \Delta\alpha_n} = \frac{2\pi \cdot L}{T \cdot \Delta\alpha_n}.$$

v = the phase velocity; L = the distance between the places of recording. $\Delta\alpha_n = \alpha_n$ for the peripheral curve— α_n for the central curve.

2. The pulse wave consists of a simple sine wave of the form men-

tioned above and a similar reflected wave with the same frequency, giving at every place of measurement a sine-formed pulse curve.

By FOURIER-analysis on a central pulse curve and a peripheral pulse curve, the phase angle difference $\Delta\alpha_n$ can be calculated. On condition that the wave is advancing (alt. 1) one gets the phase velocity from the formula given above; this velocity should be practically independent of the magnitude L . One has to reckon with a mean velocity for the distance L , however, since the qualities of the vessel (elasticity modulus, width of vessel, thickness of wall) change to a certain extent while the wave is travelling, *For purely advancing waves one will then get reciprocal like velocities independent of the magnitude of the measurement distance L .*

The situation becomes entirely different in alternative 2. If in such a case $\Delta\alpha_n$ is inserted into the formula for the phase velocities, one gets an apparent velocity which depends both on the amplitude of the pressure curve and the choice of measuring places.

At this point a retrograde wave with the form

$$p_1(x_1, t) = A_1(x) \cos n \frac{2\pi}{T} \left(t + \frac{x}{v} + t_0 \right)$$

is added to the advancing wave. Between the measuring places x_1 and x_0 one measures the phase difference $\Delta\alpha_n = \frac{2\pi}{T} \cdot \frac{L}{v} - \frac{\psi_1(x_1) - \psi(x_0)}{n}$, in which $\text{tg } \psi(x)$

$$= \frac{A_1(x) \cdot \sin n \cdot \frac{2\pi}{T} \left(\frac{2x}{v} + t_0 \right)}{A(x) + A_1(x) \cos n \frac{2\pi}{T} \left(\frac{2x}{v} + t_0 \right)}$$

from which it follows that the apparent velocity $\frac{2\pi}{\Delta\alpha_n} \cdot \frac{L}{T}$ depends on both amplitudes and choice of measuring places.

If L is altered thus one will get in calculations using the formula above the same values or at any rate reciprocal like values for v for different frequencies, if they consist of advancing waves. For frequencies which consist of an advancing wave and a reflected wave one gets the values of v_n , which are dependent on the value of L . L can be changed by, for example, moving the pulse microphone over the femoral artery peripherally or centrally in on the aorta. The latter should preferably be done on thin persons with a distinctly palpable abdominal aorta. The mathematical error when the approximation method is used is for $\Delta\alpha_1 = 2.7-8.4\%$; $\Delta\alpha_2 = 4.2-6.5\%$; $\Delta\alpha_3 = 1.6-7.2\%$. (Calculation of error carried out by Mr. BENGT ANDERSSON.)

PART III.

Experimental Section.

Technique of Investigations.

The patients lay flat on their backs on an examination table. After each patient had rested for at least 15 minutes, the examination was begun.

The pulse microphones were pressed down in the left supraclavicular fossa against the subclavian artery and in the crease of the left groin against the femoral artery, where these pulses can be palpated most easily. The microphone was fastened in the first-named position by means of an arch support and in the second position by a support made of fixed metal rods (see Fig. 2).



Fig. 2.

After every investigation the blood pressure was measured in accordance with KOROTKOFF's method. The length of the aorta (including the iliac artery) was measured according to HALLOCK's

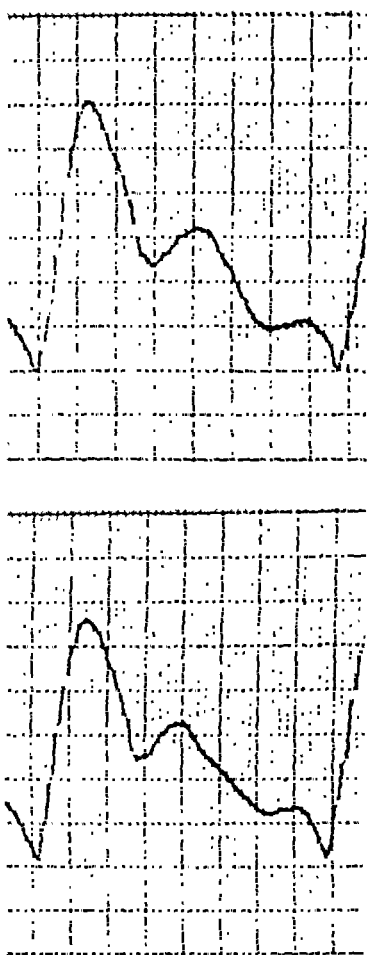


Fig. 3. Subclavian artery curves, 53 year old man. Lower curve recorded with pulse microphone pressed 3 mm deeper into the tissues than in the curve above.

method (used here in Sweden by U. CARLBORG also in his thesis). The lower edge of the insertion of the first left rib is marked on the skin. The intersection of a line marked on the skin passing over the hip bone ridges and the centre line of the body indicates the bifurcation of the aorta. The distance from the point marked at the first left rib to the bifurcation of the aorta and thence to the pulse microphone at the femoral artery is measured, and from it is deducted the distance from the pulse microphone over the

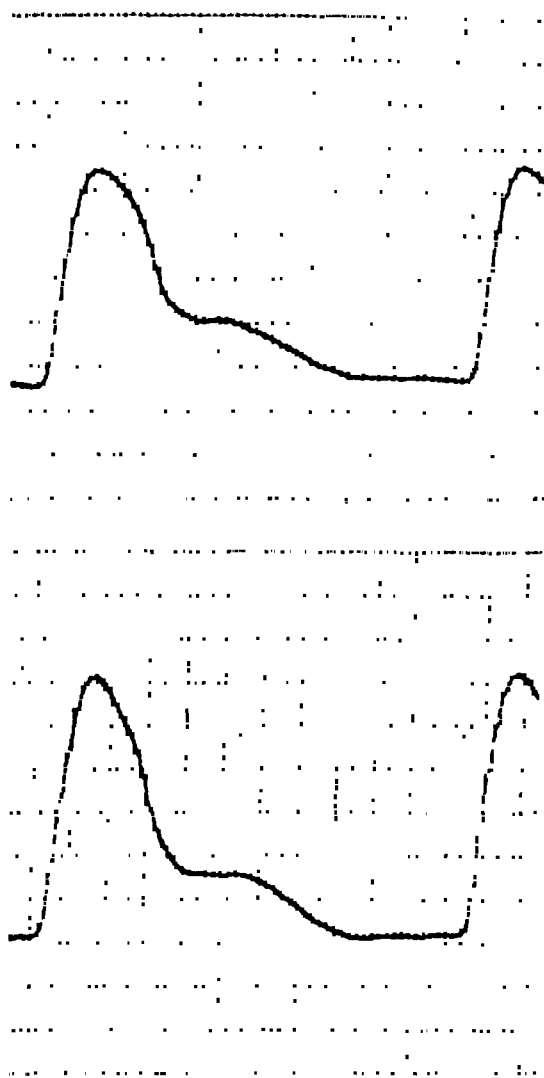


Fig. 4. Femoral artery curves, 37 year old man. Lower curve recorded with pulse microphone pressed 3 mm deeper into the tissues than in the curve above.

subclavian artery to the point by the left rib indicated above. Correction is made for the aortic arch by adding 4 cm. During the registration the patient must hold his breath.

On thin people, pulse curves were also recorded from the abdominal aorta in the same way, in order to diminish the measurement distance.

Afterwards the pulse curves were registered as in electrocardiography. The ordinate then indicated the variation in pressure and the abscissa the time (here graduated in 0.02 of a second).

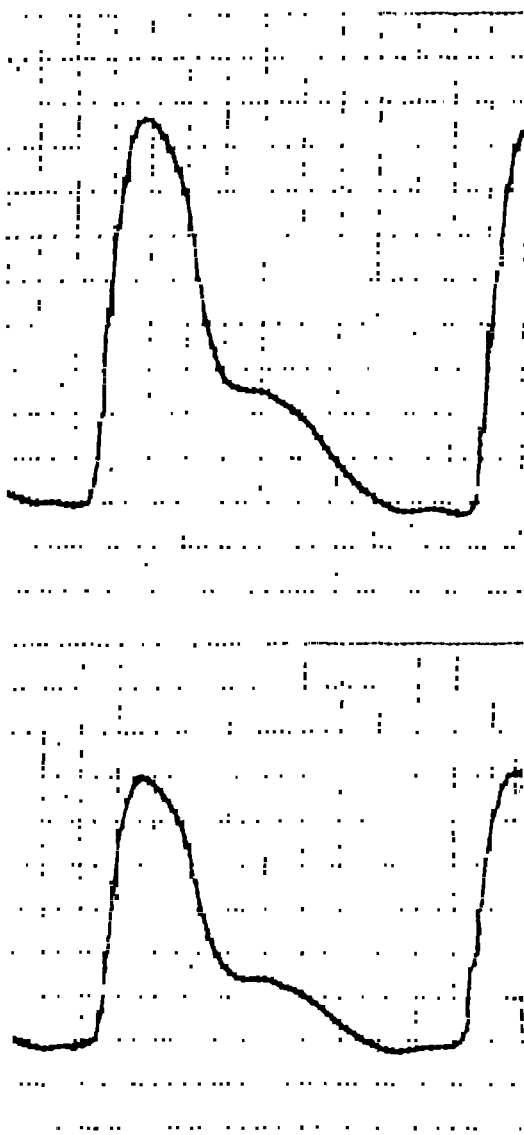


Fig. 5. Femoral artery curves, 37 year old man. Lower curve recorded with pulse microphone pressed 3 mm deeper into the tissues than in the curve above.

The time period was divided into 12 equal parts and analyzed in the way given above. The three first oscillations, which dominate powerfully, have been studied in this work.

Study of the Influence of the Measuring Apparatus on the Arterial Pulse.

Besides testing the suitability of the apparatus itself for registering the arterial pulse, I have also, as has been said, investi-

gated whether the application of the microphone affects the phenomenon to be registered. In this process the pulse microphone was first applied over the pulse (subclavian and femoral arteries) at the point where it was easiest to palpate and then pressed down within reasonable limits against the vessel. The exact depth to which the microphone was pressed down was not determined. Afterwards the pulse was registered in the manner described. Subsequently the microphone was pressed 3 mm. deeper into the tissues from this position and the pulse was recorded without changing the apparatus in any other way. One then gets practically the same form of curves or enlargement or possibly diminishment of the reading if the surface of the pulse microphone, which is affected by the pressure variation, changes. (See Fig. 3—5.) The difference is no larger than that which one finds between different periods at the same recording.

The Effects of Alteration in the Measurement Distance on the Magnitudes v_1 , v_2 and v_3 .

If the measurement distance L is altered, v_n should not, according to the theory, be altered to any extent worth mentioning if the wave is an advancing wave only. v_n is, furthermore, independent of the frequency. If, on the other hand, the wave includes a reflected portion, v_n becomes dependent on the measurement distance L .

The measurement distance has been changed by moving one (the lower) of the microphones. L has been diminished on thin patients with distinctly palpable abdominal aorta by moving the lower receptor (the "femoral receptor") over the abdominal aorta to the area around the navel, after which the subclavian artery pulse was registered synchronously with the aorta pulse near the navel. In other cases L has been extended by moving the lower microphone peripherally; thus recording was undertaken from a point on the femoral artery on the thigh. v_n was calculated afterwards according to the formula given above. The results have been tabulated. It appears from the table that in experiments 1—8, v_2 and v_3 are like one another, and that when L is changed they still have values in the same size range, while v_1 is considerably altered. According to the theory, then, the first and second partials are made up virtually of advancing waves,

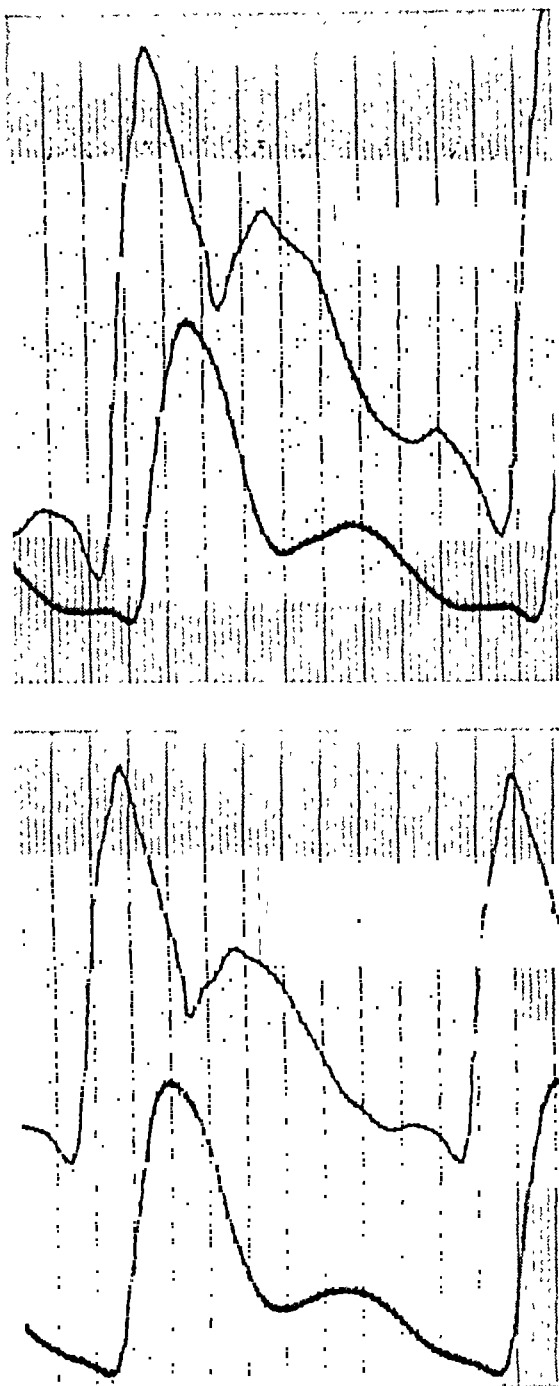


Fig. 6. Upper Figure. Upper curve: left subclavian artery; lower curve recorded from left femoral artery at the crease of the groin.

Lower Figure. Left femoral artery curve registered 11 cm. farther down the leg.

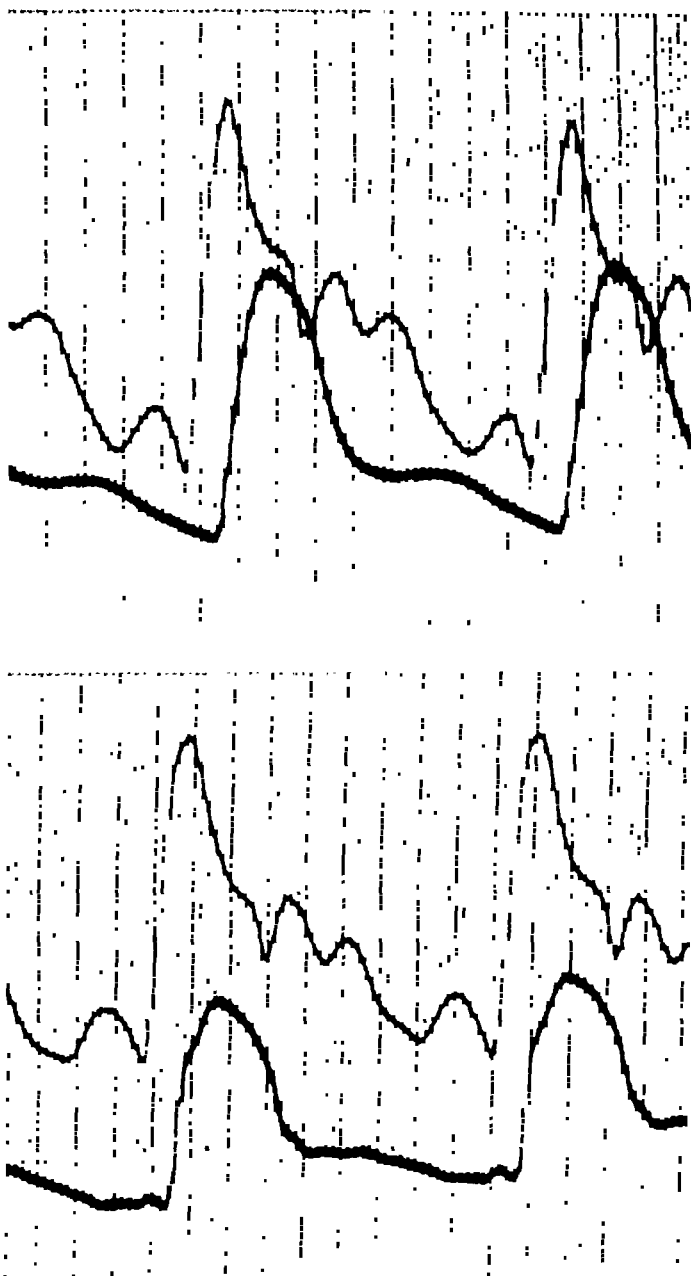


Fig. 7. Upper Figure. Upper curve: left subclavian artery; lower curve recorded from left femoral artery at the crease of the groin.

Lower Figure. Lower microphone moved to abdominal aorta by the navel.

Table 1.

Nr.	Sign.	Sex	Age	Blood Pressure in mm Hg	L	v_1	v_2	v_3
1	U-r	♂	21	140/90	51	6.7	4.0	4.1
					51	5.8	4.0	3.8
					34 _{AO} *	12.5	3.8	3.9
					34 _{AO} *	13.7	4.1	3.7
2	G-t	♂	22	130/70	49	3.5	3.5	4.7
					32 _{AO}	27.8	5.0	5.7
3	S-n	♂	26	125/80	52	8.8	5.5	4.9
					63	13.8	4.4	5.8
4	E-n	♂	26	185/85	48	42.9	4.4	5.5
					48	53.7	3.8	5.6
					58	9.4	4.7	5.8
5	E-n	♂	26	115/80	49	20.9	4.9	6.4
					49	28.0	4.8	4.8
					58	11.0	4.8	5.8
6	J-n	♂	33	115/60	48	11.8	4.0	5.0
					48	12.0	3.8	4.7
					59	5.2	3.8	5.0
					32 _{AO}	7.8	3.2	4.2
7	W-d	♂	38	115/80	48	10.8	6.2	5.5
					48	10.4	5.2	5.8
					48	9.5	5.8	6.8
					31 _{AO}	>100	6.4	6.7
					31 _{AO}	>100	5.2	6.6
8	A-d	♀	44	130/90	50	22.4	6.5	6.6
					50	15.8	7.5	8.6
					32 _{AO}	>100	5.9	5.6
9	A-g	♂	48	130/70	50	16.8	5.8	10.0
					50	19.7	5.1	8.1
					61	51.2	7.8	10.8
					61	41.0	8.5	11.1
10	E-n	♀	56	145/80	52	12.9	21.8	13.1
					52	15.1	20.6	12.7
					61	31.9	30.4	15.2

 L in m/sec. v_n in m/sec.

$v_1 > 100$ means that the phase angle difference $\Delta\alpha_n$ is very small and cannot be determined with exactitude.

v_1 is then higher than 100 m/sec.

* AO the peripheral curve is registered from the abdominal aorta.

while the fundamental vibration includes a retrograde wave. It appears from investigations of normal subjects that v_2 and v_3 are usually almost alike but different from v_1 (see table 1). This fact makes it still more likely that the fundamental vibration in these cases is reflected, while the first and second partials are virtually unreflected.

In cases 9 and 10, in which v_2 and v_3 are unlike one another, a change in L results in a considerable change in v_1 as well as v_2 , while v_3 still has the same size range. One assumes here that the fundamental vibration and the first partial are reflected, while the second partial is virtually an advancing wave.

These results strengthen further the supposition that in the first approximation one may assume that the oscillation is regulated by linear differential equations.

The illustrations show pulse curves (from the subclavian artery, abdominal aorta, and femoral artery) recorded after alteration of the measurement distance L .

Studies of the Pulse Wave in Healthy Persons.

Central (subclavian artery) and peripheral (femoral artery) pulse curves from 58 healthy persons between the ages of 20 and 78 years were recorded in the way described above. It is, of course, necessary to define "healthy" as it has been used here. At the more advanced ages the boundary between health and illness is naturally not so easy to determine. To obtain comparable material at different ages, only persons with a systolic pressure of 145—110 mm. of mercury and a diastolic pressure of 90—60 mm. of mercury were included. The following additional requirements were made of the healthy patients:

1. No vessel or heart diseases in anamnesis.
2. No current diseases.
3. Physically normal heart when examined by auscultation and percussion.
4. Normal electrocardiogram.

c_1^2 , c_2^2 , and c_3^2 and v_1 , v_2 and v_3 were calculated and tabulated. As a general rule, the calculations were made from two periods at the same recording. It appears from the table that the three first oscillations are decidedly dominant, since $c_1^2 + c_2^2 + c_3^2$ are in practically all cases ≥ 0.90 for both the central and the periph-

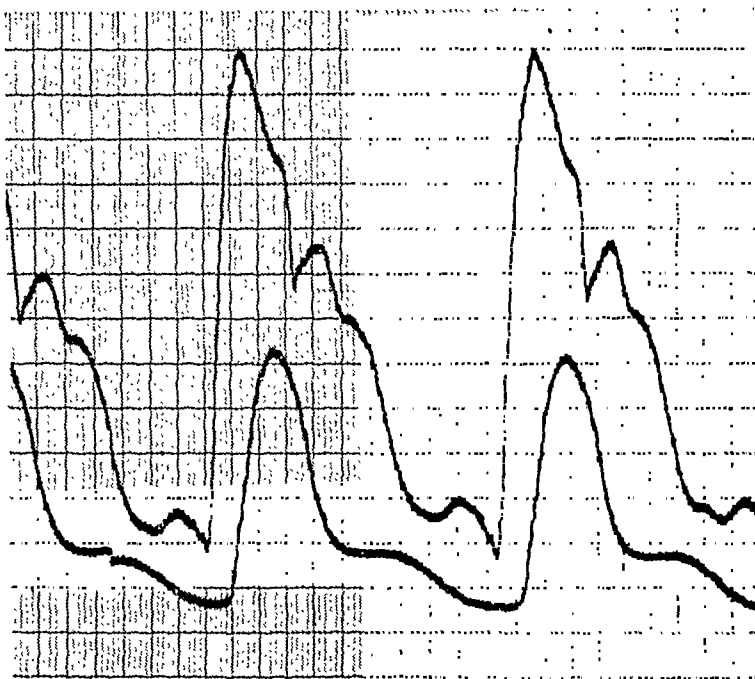


Fig. 8. Pulse curves, 34 year old healthy man. Upper curve: left subclavian artery; lower curve: left femoral artery.



Fig. 9. Pulse curves, 26 year old healthy man. Upper curve: left subclavian artery; lower curve, left femoral artery.

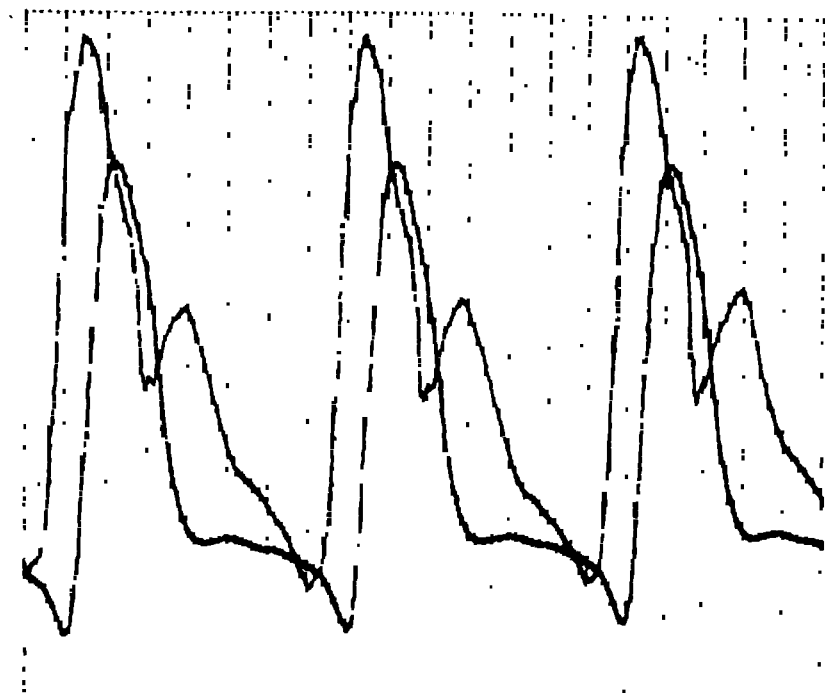


Fig. 10. Pulse curves, 41 year old healthy man. Upper curve: left subclavian artery; lower curve: left femoral artery.



Fig. 11. Pulse curves, 68 year old healthy man. Upper curve: left subclavian artery; lower curve: left femoral artery.

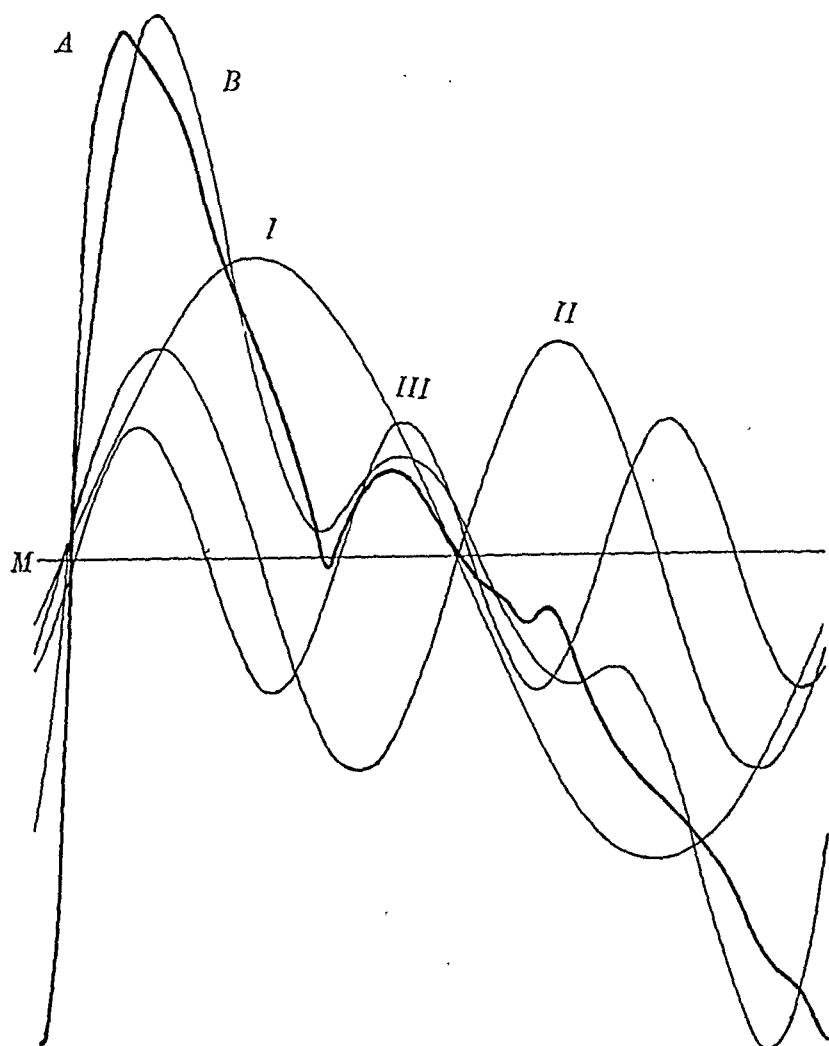


Fig. 12. A is a pulse curve (the original curve), left subclavian artery, from a 29 year old healthy woman. I is the fundamental vibration, II is the 1st partial, III is the 2nd partial. $I + II + III = B$. M = mean pulse pressure.

eral curve. These oscillations are therefore of special interest. (See fig. 12.)

If one studies the values of c_1^2 , which are put together in the tables 3—8 in age groups covering ten year periods, one finds that for both the central (subclavian artery) and peripheral (femoral artery) pulse curves, c_1^2 is greater than c_2^2 and c_3^2 ; that is to say, the fundamental vibration is decidedly dominant and thus takes up a considerable part of the pulse wave. The tables indicate that c_1^2 for the central curve varies between 0.42—0.75 in the age range 20—39 years; higher values are found only at the more advanced

ages. Thus one has here to reckon with a certain dependence on age. The corresponding values for the femoral artery are 0.50—0.80. c_2^2 is usually higher than c_3^2 . The size of c_1^2 , especially, is characteristic for different pulse curves.

If one examines more closely the magnitudes v_1 , v_2 and v_3 , which are tabulated in the same way as c , one finds that they unquestionably conform to certain laws. v_1 diverges from v_2 and v_3 and is usually considerably higher than the latter. v_2 and v_3 are, on the other hand, usually much alike and vary from 4 to 13 m/sec. According to the theory and the experiments described on p. 32, the first and second partials are formed almost entirely of advancing waves, while the fundamental vibration includes a powerful retrograde wave. This situation is characteristic of healthy subjects. v_2 and v_3 increase with age, as is clearly shown in the table. Thus the equa-

tion which on the whole is applicable to v_2 and v_3 is $v = \sqrt{\frac{\kappa}{\sigma}} = \sqrt{\frac{\delta \cdot E}{2r \cdot \sigma}}$. Since the ratio $\frac{\text{wall thickness}}{\text{radius}}$ is to be regarded as

practically constant (O. FRANK and others) and independent of the age, this increase in the speed of the advancing wave must be due to an increase in the elasticity modulus: i. e., the stretchability of the aorta diminishes with the years. Thus v_2 and v_3 become an expression of the age of the patient's vessels. Between the ages of 40 and 50 years v_2 and v_3 show a considerable increase, a fact which may be taken as clear proof of a decided aging at this period. The border values for the younger age groups lie nearer to one another than for the older, which is, indeed, to be expected, since as age increases the elasticity of the vessels is preserved more or

less well. To calculate E , $\frac{\delta}{r}$ must be known, which is not necessary in order to calculate κ , which is equal to $v^2 \cdot 1.06$. The so-called pulse wave velocity, which has been shown by earlier authors to increase with age because the time difference between the corresponding points diminishes, has no connection with the formula above, since the reflections have been ignored.

If v_1 is compared with v_2 and v_3 one finds that v_1 is usually higher and that its absolute value varies between 3.3 and 56.2 m/sec. in this material. The values of v_2 and v_3 also give information about the reflections; a considerable difference between these magnitudes argues in favour of changes in the hemodynamic relationships which diverge from the normal. The possibility of

studying the reflection relationships of the pulse wave opens a new path for diagnostics and an understanding of circulatory disturbances.

The significance of the elasticity and of the speed of the wave is made clear from the following. The aorta is regarded as an elastic tube with the cross-section surface Q and the volume elasticity modulus κ . During the systole the heart pumps in blood with the specific weight σ . The following differential equations apply to the relation between pressure and the speed of the volume flow.

$$\left\{ \begin{array}{l} -\frac{\partial p}{\partial x} = \frac{\partial i}{\partial t} \cdot \frac{\sigma}{Q} \\ -\frac{\partial i}{\partial x} = \frac{\partial p}{\partial t} \cdot \frac{Q}{\kappa} \end{array} \right. \quad (W. Weber, v. Kries)$$

x = the coordinate of length; t = the coordinate of time; p = the pressure at the root of the aorta; i = the speed of the volume flow. The turbulence is neglected. From these equations it can be deduced that an advancing pressure-flow wave moves with the speed

$v = \sqrt{\frac{\kappa}{\sigma}}$ and that p and i become proportional according to the equation:

$$p = i \cdot \frac{\sqrt{\kappa \cdot \sigma}}{Q} = i \cdot \frac{v \cdot \sigma}{Q}$$

If the blood flow from the heart to the aorta during the systole were without any reflection to speak of, the volume of blood pumped out would be:

$$\text{Volume} = V = \int_0^t i \, dt = \frac{Q}{v \cdot \sigma} \int_0^t (p - p_o) \, dt;$$

p_o = the diastolic pressure.

If the speed v increases -- i. e., if the aorta becomes more rigid, the integral $\int_0^t (p - p_o) \, dt$ must increase if the heart is to pump out the same volume. If the rigidity of the aorta is greater at the same diastolic pressure, then $\int_0^t p \, dt$, the »pressure impulse» of the heart, must also be greater in order to force out the same volume.

With p = the end systolic pressure and t = the systole time, V becomes, under the conditions given above, equal to the stroke volume. This fact opens a new way of determining the stroke volume, if the reflections are studied more closely.

Healthy Subjects.

Nr.	Age	Sex	T	Blood- Pressure in mm Hg	Arteria Subclavia				Arteria Femoralis				v_1	v_2	v_3
					c_1^2	c_2^2	c_3^2	$\sum_{n=1}^3 c_n^2$	c_1^2	c_2^2	c_3^2	$\sum_{n=1}^3 c_n^2$			
1	21	♂	0.88	110/75	0.530 0.495 * 0.588	0.254 0.260 0.301	0.134 0.164 0.112	0.918 0.919 —	0.559 0.574 0.599	0.330 0.316 0.334	0.082 0.085 0.068	0.971 0.975 —	9.3 10.2 8.2	3.8 3.7 3.6	4.9 4.8 4.7
2	21	♀	0.66	140/80	0.491 0.432	0.330 0.361	0.076 0.096	0.397 0.389	0.834 0.765	0.153 0.201	0.003 0.005	0.990 0.971	6.5 5.2	4.8 3.7	6.3 5.0
3	21	♂	1.14	120/80	0.721	0.081	0.120	0.922	0.632	0.241	0.085	0.938	12.9	4.4	4.6
4	21	♂	0.99	135/85	0.401 0.444	0.259 0.342	0.235 0.166	0.395 0.952	0.599 0.628	0.303 0.309	0.071 0.057	0.973 0.994	11.3 7.7	3.7 4.0	5.9 6.9
5	21	♂	1.05	140/90	0.532 0.583	0.182 0.189	0.207 0.172	0.921 0.944	0.559 0.554	0.296 0.313	0.116 0.106	0.971 0.973	6.7 5.8	4.0 4.0	4.1 3.8
6	22	♂	0.76	135/65	0.602	0.273	0.062	0.937	0.778	0.186	0.024	0.938	9.0	5.3	6.7
7	22	♂	0.70 0.74	130/70	0.532 0.635	0.329 0.270	0.064 0.073	0.965 0.978	0.874 0.840	0.103 0.125	0.021 0.033	0.938 0.938	3.3 3.5	3.9 3.5	2.4 4.7
8	25	♀	0.94 0.90	115/75	0.442 0.362	0.389 0.403	0.126 0.196	0.957 0.959	0.741 0.728	0.217 0.221	0.037 0.040	0.935 0.939	7.4 8.0	4.1 3.4	5.0 4.1
9	26	♂	1.08	120/80	0.458 0.435	0.275 0.287	0.210 0.215	0.943 0.937	0.693 0.659	0.256 0.267	0.039 0.055	0.933 0.931	8.8 10.5	3.5 3.6	4.3 4.7
10	26	♂	1.06 1.05	125/80	0.743 0.642	0.078 0.140	0.130 0.149	0.951 0.931	0.489 0.498	0.347 0.360	0.119 0.110	0.955 0.938	8.8 4.6	5.5 5.7	4.9 5.2
11	29	♀	0.86 0.90	115/70	0.539 0.556	0.266 0.200	0.108 0.096	0.913 0.952	0.664 0.664	0.275 0.295	0.042 0.031	0.931 0.930	11.6 20.0	3.9 4.2	4.2 4.8
12	30	♂	0.80	130/85	0.496	0.395	0.070	0.931	0.748	0.220	0.024	0.932	5.4	4.4	4.6

13	31	♀	0.68	110/75	0.505	0.845	0.092	0.942	0.714	0.232	0.032	0.978	5.9	4.2	5.0
					0.680	0.178	0.102	0.960	0.685	0.312	0.037	0.984	7.9	4.9	6.2
					0.712	0.127	0.109	0.948	0.633	0.307	0.046	0.986	5.6	4.2	4.9
14	32	♂	0.93	130/70	0.533	0.188	0.165	0.936	0.538	0.343	0.073	0.954	13.5	4.5	5.5
					0.578	0.168	0.191	0.937	0.579	0.325	0.072	0.976	7.3	3.7	4.8
15	33	♂	0.90	115/60	0.540	0.317	0.092	0.949	0.566	0.360	0.059	0.985	11.3	4.0	5.0
					0.592	0.319	0.063	0.934	0.583	0.343	0.054	0.980	12.0	3.8	4.7
16	34	♂	0.88	130/80	0.594	0.229	0.163	0.986	0.581	0.267	0.043	0.991	6.6	4.7	6.8
17	34	♂	0.97	120/80	0.577	0.139	0.212	0.928	0.531	0.347	0.072	0.950	11.1	4.8	6.4
					0.578	0.185	0.191	0.954	0.622	0.316	0.048	0.981	8.0	4.1	5.7
18	35	♂	0.80	120/80	0.664	0.268	0.055	0.987	0.671	0.304	0.019	0.994	10.3	4.5	6.9
					0.742	0.191	0.060	0.993	0.595	0.364	0.033	0.992	11.5	4.3	5.6
					0.773	0.152	0.061	0.986	0.607	0.357	0.028	0.992	12.8	4.5	5.5
19	35	♂	1.09	120/80	0.416	0.284	0.222	0.922	0.468	0.270	0.147	0.885	16.5	4.5	4.8
20	37	♂	1.20	115/75	0.538	0.065	0.191	0.794	0.544	0.255	0.128	0.927	7.7	5.2	4.1
					0.591	0.065	0.164	0.820	0.537	0.254	0.124	0.915	7.8	4.7	3.9
21	38	♂	1.01	115/80	0.745	0.118	0.109	0.972	0.611	0.251	0.113	0.975	10.8	6.2	5.5
					0.667	0.151	0.141	0.959	0.615	0.272	0.095	0.982	10.4	5.2	5.3
22	38	♂	0.81	135/85	0.506	0.394	0.053	0.953	0.764	0.208	0.019	0.991	6.7	4.6	5.6
					0.483	0.406	0.041	0.930	0.803	0.171	0.010	0.989	5.6	4.2	5.9
23	39	♂	0.93	115/80	0.638	0.168	0.123	0.973	0.588	0.325	0.060	0.973	22.8	5.9	6.3
					0.638	0.202	0.133	0.973	0.594	0.325	0.054	0.973	18.2	5.0	6.7
24	39	♀	0.95	115/85	0.570	0.139	0.211	0.920	0.687	0.262	0.042	0.991	9.7	3.9	4.6
					0.571	0.163	0.191	0.915	0.693	0.264	0.035	0.992	6.4	3.8	4.5
25	40	♂	0.90	120/70	0.612	0.166	0.154	0.982	0.596	0.326	0.057	0.979	9.5	5.3	6.1
					0.566	0.186	0.172	0.924	0.597	0.306	0.067	0.970	10.1	5.5	6.6
26	40	♀	0.61	135/85	0.599	0.341	0.042	0.982	0.678	0.273	0.032	0.983	10.5	6.6	8.4
					0.570	0.376	0.039	0.985	0.710	0.252	0.024	0.985	8.9	6.2	8.8
					0.551	0.363	0.070	0.984	0.642	0.282	0.052	0.976	7.8	5.6	8.2
27	42	♀	0.72	120/75	0.687	0.174	0.109	0.970	0.663	0.245	0.061	0.969	13.1	6.2	7.1
					0.674	0.185	0.111	0.970	0.655	0.262	0.057	0.974	12.4	6.4	6.9

* Computed with aid of harmonic analyzer.

Nr.	Age	Sex	T	Blood- Pressure in mm Hg	Arteria Subclavia				Arteria Femoralis				v_1	v_2	v_3
					c_1^2	c_2^2	c_3^2	$\sum_{n=1}^3 c_n^2$	c_1^2	c_2^2	c_3^2	$\sum_{n=1}^3 c_n^2$			
28	42	♂	0.95	130/90	0.809 0.854	0.104 0.089	0.060 0.041	0.973 0.984	0.620 0.645	0.264 0.254	0.082 0.079	0.966 0.978	38.8 48.2	10.7 14.3	6.1 5.9
29	44	♀	0.73 0.76	130/90	0.658 0.734	0.190 0.169	0.140 0.067	0.988 0.970	0.679 0.665	0.275 0.275	0.020 0.033	0.974 0.973	22.4 15.8	6.5 7.5	6.6 8.6
30	44	♂	0.91 0.94	125/85	0.780 0.799	0.151 0.116	0.059 0.049	0.990 0.964	0.640 0.662	0.236 0.238	0.081 0.070	0.957 0.970	24.8 23.9	11.3 6.3	8.2 7.3
31	46	♂	0.87	130/80	0.699	0.264	0.107	0.970	0.624	0.313	0.051	0.988	10.3	4.7	5.9
32	48	♂	0.86 0.89	120/75	0.761 0.778	0.127 0.143	0.083 0.059	0.971 0.980	0.617 0.612	0.316 0.323	0.052 0.054	0.985 0.989	51.2 41.0	7.3 8.6	10.8 11.1
33	48	♀	0.97 0.86	120/75	0.844 0.836	0.094 0.103	0.040 0.040	0.978 0.979	0.590 0.598	0.326 0.291	0.062 0.074	0.978 0.963	27.6 11.4	6.3 6.0	7.2 8.5
34	48	♂	0.83 0.83	130/80	0.696 0.719	0.116 0.112	0.161 0.150	0.973 0.981	0.670 0.673	0.255 0.257	0.059 0.057	0.984 0.987	7.4 8.3	5.3 5.0	6.0 6.2
35	50	♂	0.90 0.92	145/95	0.788 0.798	0.109 0.127	0.063 0.111	0.960 0.946	0.710 0.643	0.198 0.258	0.069 0.069	0.977 0.970	10.5 26.8	6.4 6.9	8.1 8.7
36	50	♂	0.83	130/90	0.591	0.329	0.052	0.972	0.713	0.219	0.044	0.976	56.2	6.2	7.7
37	50	♀	0.77	135/80	0.702	0.225	0.052	0.979	0.727	0.219	0.025	0.971	15.7	5.7	7.7
38	51	♂	0.85 0.88	140/90	0.723 0.749	0.131 0.082	0.100 0.130	0.954 0.961	0.619 0.594	0.290 0.306	0.064 0.075	0.973 0.975	9.2 11.5	5.6 5.4	6.0 6.1
39	52	♂	0.67 0.66	135/90	0.663 0.619	0.262 0.285	0.056 0.068	0.981 0.962	0.710 0.708	0.239 0.233	0.040 0.040	0.989 0.981	13.4 10.5	7.7 6.5	10.8 9.0
40	53	♂	0.83 0.85	130/75	0.645 0.706	0.179 0.238	0.141 0.002	0.965 0.946	0.697 0.699	0.234 0.265	0.045 0.002	0.076 0.966	11.1 14.4	5.5 5.3	7.1 7.8
41	53	♂	1.08	140/90	0.735	0.139	0.078	0.952	0.555	0.232	0.143	0.930	9.3	8.6	5.2

42	54	♂	1.07	0.723	0.115	0.098	0.996	0.533	0.235	0.156	0.924	6.5	10.5	4.6
43	55	♀	1.06	0.748	0.130	0.079	0.957	0.577	0.257	0.113	0.947	8.1	8.5	5.5
44	56	♀	0.94	0.771	0.108	0.072	0.951	0.616	0.253	0.087	0.956	7.7	7.1	6.3
45	56	♂	0.96	0.716	0.135	0.106	0.957	0.638	0.263	0.070	0.971	37.6	7.5	6.7
46	57	♀	0.63	0.912	0.076	0.094	0.992	0.787	0.186	0.020	0.993	12.9	21.3	13.1
47	57	♂	0.65	0.859	0.120	0.014	0.993	0.781	0.191	0.020	0.992	15.1	20.6	12.7
48	60	♂	0.98	0.570	0.142	0.190	0.902	0.625	0.242	0.095	0.962	5.9	6.1	5.1
49	63	♂	0.93	0.553	0.123	0.220	0.896	0.621	0.233	0.088	0.962	8.7	6.2	5.2
50	64	♂	0.91	0.848	0.131	0.009	0.988	0.676	0.236	0.067	0.979	18.9	8.7	6.4
51	65	♂	0.95	0.817	0.107	0.056	0.980	0.660	0.250	0.059	0.969	10.8	9.7	7.2
52	65	♂	0.93	0.860	0.084	0.039	0.983	0.668	0.257	0.055	0.980	11.6	8.9	7.2
53	66	♂	0.66	0.675	0.233	0.070	0.978	0.735	0.176	0.020	0.981	11.1	5.6	9.9
54	66	♂	0.67	0.640	0.255	0.038	0.983	0.735	0.221	0.028	0.984	10.1	5.2	9.0
55	70	♂	0.90	0.813	0.136	0.033	0.981	0.720	0.205	0.055	0.980	22.5	16.0	15.6
56	72	♂	0.87	0.848	0.107	0.022	0.977	0.751	0.188	0.047	0.986	23.2	15.0	13.3
57	78	♂	1.09	0.777	0.146	0.040	0.963	0.640	0.208	0.109	0.952	15.2	21.7	9.3
58	78	♂	1.07	0.803	0.133	0.040	0.976	0.603	0.208	0.126	0.937	19.4	17.2	8.8
59	85	♂	0.95	0.769	0.135	0.062	0.966	0.711	0.208	0.059	0.978	11.8	10.4	8.1
60	85	♂	0.92	0.759	0.139	0.079	0.977	0.725	0.210	0.051	0.986	11.6	9.5	7.9
61	85	♂	0.80	0.824	0.104	0.066	0.994	0.753	0.216	0.026	0.995	14.7	11.0	8.8
62	86	♂	0.80	0.817	0.132	0.045	0.994	0.751	0.214	0.029	0.994	17.0	14.2	10.5
63	86	♂	0.95	0.715	0.133	0.070	0.968	0.698	0.235	0.051	0.984	23.2	8.8	8.3
64	86	♂	0.78	0.528	0.267	0.134	0.979	0.672	0.248	0.064	0.984	12.9	5.4	8.0
65	87	♂	0.84	0.520	0.161	0.198	0.879	0.633	0.261	0.079	0.973	10.3	6.3	7.3
66	87	♂	0.80	0.765	0.147	0.066	0.978	0.684	0.237	0.057	0.978	51.6	34.6	11.5
67	90	♂	0.84	0.949	0.021	0.032	0.992	0.794	0.169	0.025	0.988	13.9	24.7	20.9
68	90	♂	0.83	0.852	0.114	0.025	0.991	0.806	0.157	0.027	0.990	11.9	16.1	16.1
69	90	♂	0.80	0.541	0.137	0.236	0.963	0.688	0.231	0.059	0.978	7.0	6.4	7.8
70	90	♂	1.10	0.747	0.182	0.054	0.983	0.675	0.211	0.080	0.966	19.2	9.6	7.7
71	90	♂	1.10	0.765	0.161	0.053	0.979	0.688	0.213	0.072	0.973	21.8	10.6	7.6

Table 3.
 c_1^2 *Arteria subclavia.*

Age	Number of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	σ log	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20—29	11	-0.268 ± 0.0251	0.540	0.0834	0.10	19.2	0.401—0.743
30—39	13	-0.238 ± 0.0191	0.579	0.0689	0.09	15.9	0.416—0.745
40—49	10	-0.155 ± 0.0175	0.700	0.0553	0.09	12.7	0.599—0.844
50—59	13	-0.141 ± 0.0817	0.723	0.0642	0.11	14.8	0.570—0.912
60—69	8	-0.138 ± 0.0221	0.728	0.0626	0.10	14.4	0.528—0.824
70—	3	-0.139 ± 0.0763	0.727				

Table 4.
 c_2^2 *Arteria subclavia.*

Age	Number of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	σ log	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20—29	11	-0.654 ± 0.0711	0.222	0.236	0.12	54.3	0.078—0.389
30—39	13	-0.704 ± 0.0626	0.198	0.226	0.10	52.0	0.065—0.395
40—49	10	-0.797 ± 0.0566	0.160	0.179	0.07	41.2	0.094—0.341
50—59	13	-0.832 ± 0.0490	0.147	0.177	0.06	40.8	0.076—0.329
60—69	8	-0.792 ± 0.0477	0.162	0.135	0.05	31.1	0.104—0.267
70—	3	-1.093 ± 0.2970	0.081				

Table 5.
 c_3^2 *Arteria subclavia.*

Age	Number of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	σ log	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20—29	11	-0.921 ± 0.0648	0.120	0.215	0.06	49.5	0.054—0.235
30—39	13	-0.914 ± 0.0626	0.122	0.226	0.06	52.0	0.053—0.222
40—49	10	-1.070 ± 0.0725	0.085	0.229	0.04	52.7	0.040—0.161
50—59	13	-1.270 ± 0.1280	0.054	0.461	0.06	106.1	0.004—0.190
60—69	8	-1.186 ± 0.0809	0.065	0.229	0.03	52.7	0.033—0.184
70—	3	-1.157 ± 0.3290	0.070				

Table 6.
 c_1^2 *Arteria femoralis.*

Age	Num- ber of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	σ log	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20-29	11	-0.177 ± 0.0076	0.666	0.025	0.04	5.8	0.489-0.874
30-39	13	-0.213 ± 0.0175	0.613	0.063	0.09	14.6	0.468-0.764
40-49	10	-0.196 ± 0.0072	0.637	0.023	0.03	5.2	0.590-0.679
50-59	13	-0.174 ± 0.0110	0.670	0.040	0.06	9.1	0.555-0.787
60-69	8	-0.151 ± 0.0097	0.707	0.028	0.04	6.3	0.640-0.785
70-	3	-0.144 ± 0.0069	0.718				

Table 7.
 c_2^2 *Arteria femoralis.*

Age	Num- ber of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	σ log	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20-29	11	-0.632 ± 0.0479	0.234	0.159	0.09	36.6	0.103-0.347
30-39	13	-0.553 ± 0.0221	0.280	0.080	0.05	18.4	0.208-0.360
40-49	10	-0.551 ± 0.0167	0.281	0.053	0.03	12.2	0.236-0.326
50-59	13	-0.631 ± 0.0139	0.234	0.050	0.03	11.6	0.186-0.290
60-69	8	-0.668 ± 0.0170	0.215	0.047	0.02	10.9	0.176-0.248
70-	3	-0.695 ± 0.0410	0.202				

Table 8.
 c_3^2 *Arteria femoralis.*

Age	Num- ber of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	σ log	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20-29	11	-1.383 ± 0.138	0.041	0.458	0.04	105.5	0.003-0.119
30-39	13	-1.280 ± 0.082	0.053	0.296	0.04	68.2	0.019-0.147
40-49	10	-1.284 ± 0.060	0.052	0.190	0.02	43.7	0.020-0.082
50-59	13	-1.249 ± 0.065	0.056	0.234	0.03	53.9	0.020-0.143
60-69	8	-1.308 ± 0.082	0.049	0.231	0.03	53.2	0.020-0.109
70-	3	-1.309 ± 0.153	0.049				

Table 9.

 v_1 .

Age	Number of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	$\sigma \log$	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20—29	11	0.915 ± 0.047	8.22	0.157	2.97	36.2	3.3—11.6
30—39	13	1.000 ± 0.047	12.60	0.168	4.87	38.7	5.4—22.8
40—49	10	1.350 ± 0.096	13.65	0.309	9.71	71.1	7.4—51.2
50—59	13	1.133 ± 0.075	12.98	0.269	8.04	61.9	5.9—56.2
60—69	8	1.251 ± 0.073	13.34	0.207	6.36	47.7	11.1—51.6
70—	3	1.090 ± 0.098					7.0—19.2

Table 10.

 v_2 .

Age	Number of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	$\sigma \log$	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20—29	11	0.625 ± 0.0194	4.22	0.0643	0.62	14.8	3.5—5.5
30—39	13	0.675 ± 0.0160	4.73	0.0577	0.63	13.3	3.9—6.2
40—49	10	0.829 ± 0.0389	6.75	0.123	1.91	28.3	4.7—11.3
50—59	13	0.879 ± 0.0438	7.57	0.158	2.75	36.4	5.5—21.3
60—69	8	1.070 ± 0.0990	12.80	0.281	8.28	64.7	5.4—34.6
70—	3	1.060 ± 0.1700	12.77				

Table 11.

 v_3 .

Age	Number of cases	Mean $\pm \Sigma_m$ (log)	Mean abs.	$\sigma \log$	Standard deviation		Range founded values
					σ_x abs.	percent of mean	
20—29	11	0.672 ± 0.036	4.70	0.120	1.30	27.6	2.4—6.7
30—39	13	0.742 ± 0.021	5.52	0.075	0.95	17.2	4.1—6.9
40—49	10	0.852 ± 0.003	7.12	0.080	1.31	18.4	5.9—10.8
50—59	13	0.859 ± 0.032	7.23	0.115	1.91	26.5	5.1—13.1
60—69	8	0.987 ± 0.032	9.71	0.092	2.05	21.1	8.0—15.6
70—	3	1.033 ± 0.144	12.69				

Experimental Errors and Statistics.

In order to determine the errors involved in the method used for estimation of the velocities (v_n) and amplitude-squares (c_n^2), double determinations have been performed in 40 healthy subjects of different age. These magnitudes are computed from two pulse-periods at the same experiment. The experimental error thus means the error of *two* determinations and involves the approximation used for calculation of the different magnitudes, the error of measuring, and the biological variation between two pulse-periods.

From the table 12 it is obvious that the determination of c_1^2 and c_2^2 in arteria femoralis have a rather small experimental error, 2—4 %, and likewise the determination of c_1^2 in arteria subclavia, 3.55 %.

It is inherent in the method that the other magnitudes c_3^2 and c_4^2 , will have a somewhat greater experimental error: 10—14 %. v_1 has a rather great experimental error (19 %) due to its calculation from a less phase angle difference, v_2 and v_3 have a small experimental error 8—13 %.

The magnitudes of the experimental errors are what is to be expected from a method working on biological material and involving the variation between two biological magnitudes, as the experimental error in these case comprises the errors of the apparatus, of the calculation and of the biological variability between two pulse-periods. CROZIER, WOLF and ZERRAHN-WOLF (1937) (cit. L. GOLDBERG) stated that the value of the percentage error of a function are of about the same order of magnitude in very different sensory effects. CROZIER and HOLWAY (1940) (cit. L. GOLDBERG) consider the percentage standard deviation to be constant and a property of the reacting organism; they found for different functions 10—15 %. GOLDBERG (1943) has shown the same thing for tests of sensory, motor and physiological functions, and found the percentage standard deviation to vary between 5 and 20 % in 7 different tests. (Acta Physiol. Scand. 1943, 5. Suppl. XVI.)

Statistics: n = number of cases x = single value $S(\quad)$ = number of ... \bar{x} = mean of x values $= \frac{S(x)}{n}$ σ_x = standard deviation of x -values $= \pm \sqrt{\frac{S(x - \bar{x})^2}{n - 1}}$ $\varepsilon_{\bar{x}}$ = standard error of mean $= \frac{\sigma_x}{\sqrt{n}}$ $\sigma_x\%$ = percentage standard deviation of x values, expressed
in per cent of mean $= \frac{\sigma_x \cdot 100}{\bar{x}}$

If standard deviation is computed from logarithms ($\log \sigma$), σ_x is expressed in per cent of mean by the following formula: (COCHRAN, 1938) (cit. L. GOLDBERG), which is rather accurate up to standard deviations of 20–40 %:

 σ_x in percent $= 230.26 \cdot \log \sigma$.**Double determinations:** d = difference between two values. \bar{d} = mean difference $= \frac{S(d)}{n}$ σ_d = standard deviation of differences $= \pm \sqrt{\frac{S(d - \bar{d})^2}{n - 1}}$ $\varepsilon_{\bar{d}}$ = standard error of mean difference $= \frac{\sigma_d}{\sqrt{n}}$

The experimental error of the method (σ_d), *i. e.* the error of a single value, can be calculated by the following formula, if the mean difference (\bar{d}) is less than three times its standard error ($\bar{d} < 3 \cdot \varepsilon_{\bar{d}}$)

$$\sigma_x = \frac{1}{\sqrt{2}} \cdot \sigma_d$$

The values found of v_n and c_n^2 for the different age groups show in most groups a very marked skew distribution (tables 3–11).

Table 12.

Double determinations of v_n and c_n^2 in 40 healthy subjects.

	Number of cases	Mean of first values	Difference between first and second value	Standard devia- tion of diff. σ_d	Standard deviation of single value		Error of double deter- mina- tions in percent of mean
					absolute value	percent of mean	
					$\sigma_x = \frac{1}{\sqrt{2}} \cdot \sigma_d$	$\sigma_x \cdot \frac{100}{x}$	
	1	2	3	4	5	6	
A. subclavia c_1^2	40	0.656	-0.0062 ± 0.0075	0.047	0.033	5.03	3.55
c_2^2	40	0.188	0.0028 ± 0.0062	0.039	0.028	14.89	10.53
c_3^2	40	0.109	0.0028 ± 0.0048	0.031	0.022	20.18	14.26
A. femoralis c_1^2	40	0.660	-0.0044 ± 0.0052	0.033	0.023	3.48	2.46
c_2^2	40	0.256	0.0069 ± 0.0035	0.022	0.016	6.25	4.41
c_3^2	40	0.056	0.0 ± 0.019	0.122	0.086	15.35	10.35
v_1	40	13.46	-0.443 ± 0.802	5.07	3.58	26.59	18.80
v_2	40	7.08	-0.385 ± 0.288	1.82	1.28	18.20	12.87
v_3	40	7.05	-0.218 ± 0.190	1.20	0.85	12.05	8.52

When transformed to logarithms the values within each age group show a rather symmetrical normal distribution. This was shown by graphical representation according to BLISS (1938) (cit. L. GOLDBERG), there the additive percentual distributions are transformed to probability units and plotted against the logarithms of the values found. The resulting graph becomes a straight line if the logarithmic values are normally distributed. The agreement with expectation was good.

The logarithmic standard deviation explains why the percentage standard deviation can become $> 100\%$.

Up to the age of 40 years the standard deviations generally are of a magnitude from 5—50 %, which is to be expected from a biological population for the variability between different individuals.

In ages over 40 years the percentage standard deviation as a rule increased which among other things can depend on these age groups not being absolutely homogeneous as to healthy subjects but comprising possibly individuals with slight pathological disturbances.

Clinical Studies.

The Character of the Pulse Wave in Cases of High Blood Pressure.

In order to obtain a general idea of the nature of the pulse wave in cases of high blood pressure, pulse curves from a number of hypertensive patients of different ages and with varying clinical symptoms have been recorded and analyzed. (See fig. 13—14.) The information thus obtained throws light upon the serviceableness of the methodics and will be followed by a more systematic investigation of hypertensive diseases. The systolic blood pressure has ranged from 150 to 250 mm. of mercury, and the diastolic blood pressure from 90 to 140 mm. of mercury. The ages have ranged from 29 to 75 years.

The results have been tabulated in table 13. In the greater number of cases, c_1^2 is higher than in healthy subjects for both the central and the peripheral curves (no. 1 is an exception). Thus the fundamental vibration dominates more powerfully than in healthy subjects: *the wave is of another type.*

If the v_n magnitudes are examined, one finds that v_2 and v_3 are higher than in healthy persons of the same age. This is probably to be ascribed to an increase in the modulus of the vessel wall. This investigation does not reveal whether the increase in the elasticity modulus is due to the fact that an increase in the mean pressure primarily reduces the stretchability of the vessel wall or to the fact that the wall is pathologically changed. Remarkably high values at relatively low pressure indicate changes in the vessel wall due to advanced arteriosclerosis. In cases 2 and 3 there were severe clinical symptoms consisting of difficulty with decompensation, pounding heart, etc., and changes in the eyegrounds. In these cases the values of v_n indicate increased reflections; both the fundamental vibration and the first partial are powerfully reflected. At present the reason for this change in the hemodynamics cannot be determined with certainty. Probably the increased reflections are brought about by changes in the resistance of the peripheral vessels.

Further studies making use of agents which increase the blood pressure can probably provide an answer to this question.

Thus in a number of hypertensive cases the pulse wave has a different character than it has in healthy persons, with a more dom-



Fig. 13. Pulse curves from a 35 year old hypertensive man — blood pressure 220/140 mm Hg. Upper curve: left subclavian artery; lower curve: left femoral artery. Case Nr 2.

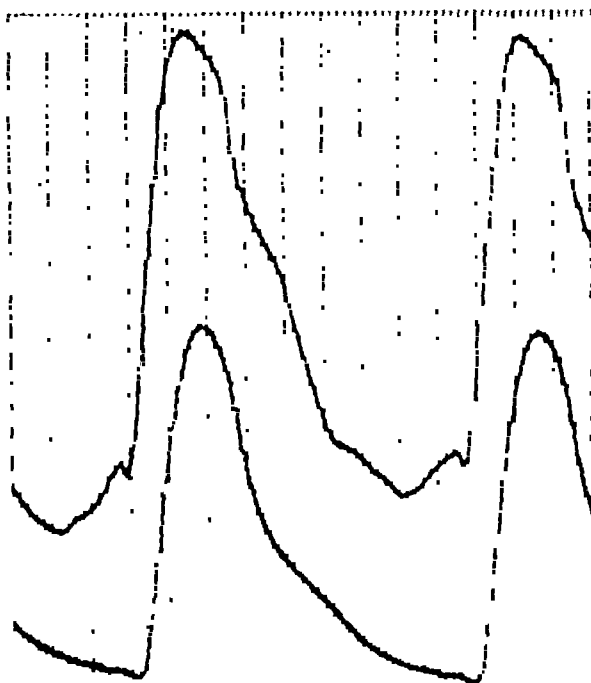


Fig. 14. Pulse curves from a 36 year old hypertensive woman. Blood pressure 250/130 mm Hg. Upper curve: left subclavian artery; lower curve: left femoral artery. Case Nr 3.

Table 13.
Cases of High Blood Pressure.

Nr	Age	Sex	T.	Blood- Pressure in mm Hg	Arteria Subclavia				Arteria Femoralis				r_1	v_2	v_3	
					c_1^2	c_2^2	c_3^2	$\sum_{n=1}^3 c_n^2$	c_1^2	c_2^2	c_3^2	$\sum_{n=1}^3 c_n^2$				
1	29	♂	0.85 0.86	150/115	0.588 0.573	0.069 0.155	0.305 0.220	0.962 0.948	0.664 0.682	0.268 0.246	0.061 0.062	0.988 0.990	13.7 11.4	6.7 8.2	6.1 7.2	
2	35	♂	0.80	220/140	0.792 0.750	0.134 0.156	0.065 0.083	0.992 0.989	0.730 0.721	0.214 0.208	0.042 0.050	0.986 0.979	22.9 23.0	18.0 17.4	10.1 10.5	Fundus Hyper- tonicus I.
3	36	♀	0.88 0.87	250/130	0.857 0.856	0.101 0.096	0.033 0.039	0.991 0.991	0.751 0.755	0.181 0.186	0.031 0.053	0.983 0.994	17.1 31.9	19.8 15.9	10.9 10.6	Fundus Hyper- tonicus III.
4	39	♀	0.80 0.70	220/140	0.861 0.842	0.108 0.125	0.016 0.012	0.985 0.979	0.773 0.791	0.189 0.183	0.025 0.012	0.987 0.986	10.3 12.9	7.5 8.7	7.4 8.3	
5	42	♂	0.76 0.79	200/120	0.744 0.750	0.187 0.178	0.045 0.050	0.976 0.978	0.742 0.737	0.222 0.220	0.022 0.024	0.986 0.981	11.0 10.6	8.1 9.1	11.0 9.6	
6	45	♀	0.69 0.70	175/90	0.760 0.711	0.197 0.241	0.025 0.033	0.983 0.985	0.819 0.805	0.149 0.160	0.017 0.016	0.985 0.981	20.5 21.9	8.0 8.5	10.0 11.1	
7	61	♀	0.79	170/95	0.810	0.141	0.037	0.988	0.760	0.188	0.022	0.970	10.1	11.0	17.6	
8	62	♂	0.70	150/80	0.781	0.150	0.050	0.981	0.782	0.191	0.010	0.983	11.2	15.1	13.8	
9	64	♀	1.06	165/90	0.855 0.812 0.850	0.124 0.147 0.135	0.011 0.025 0.014	0.996 0.984	0.797 0.814 0.815	0.167 0.158 0.162	0.030 0.024 0.023	0.994 0.996	12.6 11.7 13.6	9.6 14.5 12.7	8.6 10.2 11.5	* Computed with aid of harmonic analyzer.
10	75	♀	0.88	160/90	0.836	0.117	0.024	0.977	0.734	0.192	0.043	0.969	26.5	47.3	45.5	

inant fundamental vibration. The reflections may be different and the speed of transmission (the speed of the advancing wave) may be higher.

Studies in Cases of Coarctation of the Aorta (Isthmus Stenosis).

The nature of the pulse wave in cases of coarctation of the aorta (isthmus stenosis) is of the greatest interest from the theoretical hemodynamical, clinical, and diagnostic points of view. How a constriction changes a wave movement is well known from acoustics and hydrodynamics. Theoretically one may expect the following:

1. The occurrence of stenosis brings with it a considerable damping of both the mean pressure and the oscillating pressure.
2. Stenosis brings with it abnormal reflections, with a tendency to the formation of standing waves in front of the place of stenosis.
3. Stenosis brings with it a phase lag. The wave can naturally be reflected farther along, after which it is reflected back and forth.

The condition mentioned in 1. has been used in the diagnostics. As is known, low blood pressure and low blood pressure amplitude in the lower extremities draw the clinician's attention to the diagnosis isthmus stenosis. In Sweden B. EJRUP has carried out systematic oscillometric examinations of a large number of cases. For reasons easy to understand, the reflection relationships of the pulse wave have not been studied earlier.

The author has had the opportunity to study four cases of certain clinical isthmus stenosis. In two cases (nos. 1 and 2) the diagnosis has been verified by operation. Furthermore, all of them showed all the clinical and roentgenologic symptoms of isthmus stenosis: high blood pressure in the arms, low blood pressure and small blood pressure amplitude in the lower extremities, and erosions at the lower borders of the ribs. The blood pressure in the lower extremities has been measured oscillometrically and above the ankles. Such measurements cannot, of course, be exact, but they afford some guidance.

Pulse curves have been traced in the manner indicated above. The pictures show the appearance of the pulse curves. c_1^2 , c_2^2 , c_3^2 , v_1 , v_2 , and v_3 have been calculated and assembled in tabular form. It appears clearly from the table that c_1^2 is higher than in

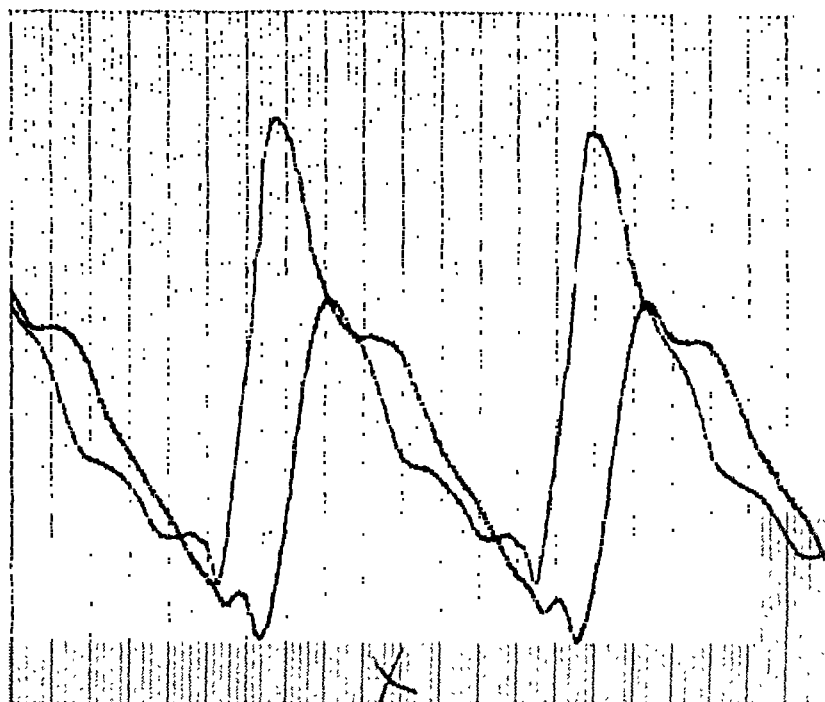


Fig. 15. Case Nr. 1. Diagnosis: isthmus stenosis, 17 year old man. Upper curve: left subclavian artery; lower curve: left femoral artery.

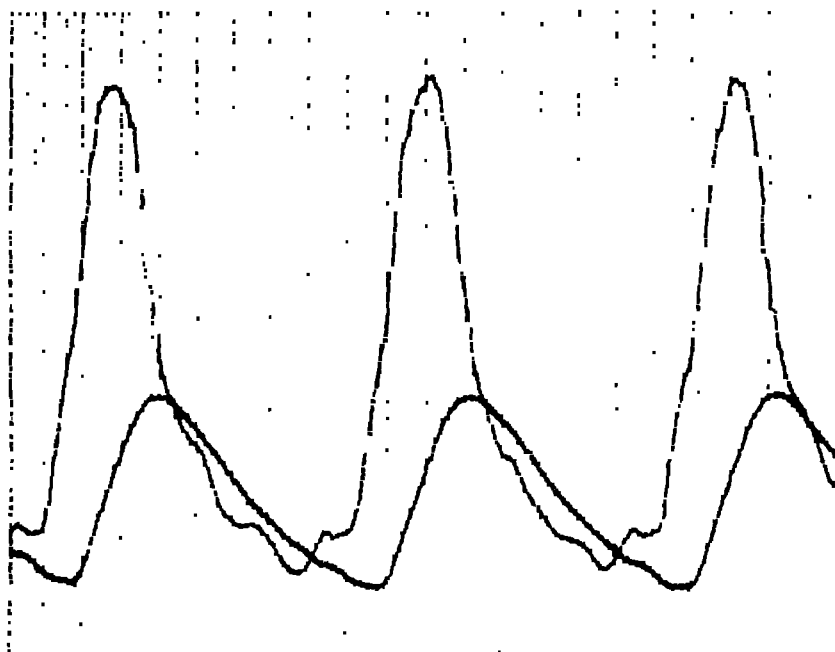


Fig. 16. Case Nr. 2. Diagnosis: isthmus stenosis, 24 year old man. Upper curve: left subclavian artery; lower curve: left femoral artery.

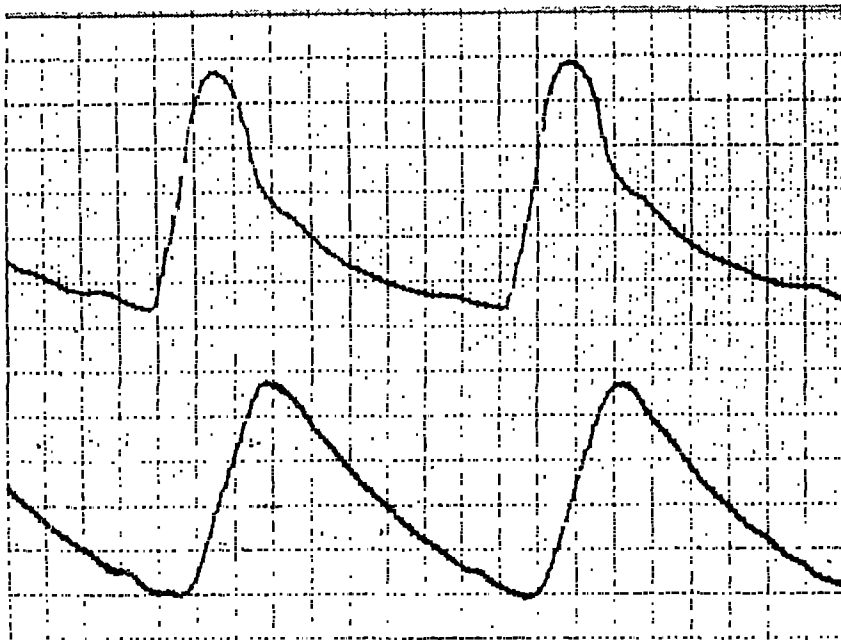


Fig. 17. Case Nr. 3. Diagnosis: isthmus stenosis; 28 year old woman. Upper curve: left subclavian artery; lower curve: left femoral artery.

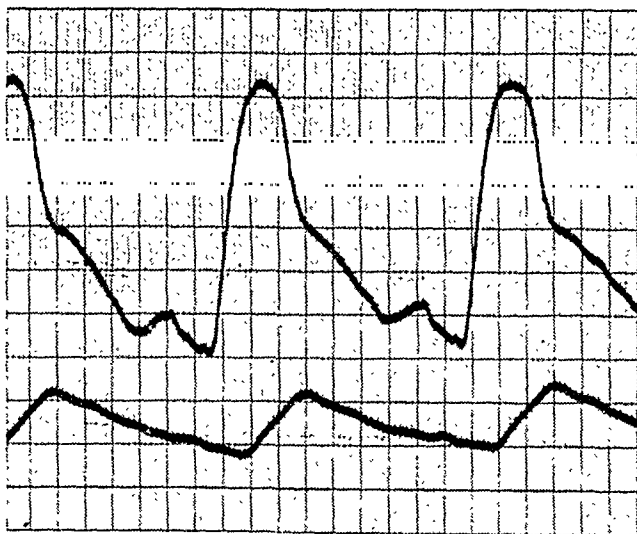


Fig. 18. Case Nr. 4. Diagnosis: isthmus stenosis; 39 year old woman. Upper curve: left subclavian artery; lower curve: left femoral artery.

healthy subjects for both the central and the peripheral curves. Thus the fundamental vibration dominates, *particularly in the peripheral curve (femoral artery), in which the pulse curve approaches the pure sine curve.* This circumstance should indicate that the

Tabla 14.
Cases of Coarctation of the Aorta

Nr	Age	Sex	T	Blood-Pressure in mm Hg				Arteria Subclavia				Arteria Femoralis				v_1	v_2	v_3
				Arm.		Leg.		c_1^2	c_2^2	c_3^2	$\sum_{n=1}^3 c_n^2$	c_1^2	c_2^2	c_3^2	$\sum_{n=1}^3 c_n^2$			
				Right	Left	Right	Left											
1	17	♂	0.84 0.83	180/80?	200/80?	105/?	110/?	0.764 0.770	0.177 0.176	0.040 0.037	0.981 0.988	0.868 0.887	0.095 0.082	0.022 0.024	0.985 0.993	2.5 2.7	3.5 3.7	4.2 4.5
2	24	♂	0.81 0.80	150/60	130/90	130/110	100/80	0.791 0.846	0.169 0.145	0.030 0.006	0.990 0.997	0.918 0.916	0.071 0.076	0.008 0.007	0.997 0.999	3.7 4.1	5.2 4.9	9.8 11.8
3	28	♀	0.93 0.96	140/?	170/90	?	140/?	0.695 0.717	0.206 0.202	0.084 0.065	0.985 0.984	0.892 0.859	0.103 0.121	0.004 0.019	0.999 0.999	3.1 3.1	4.1 3.6	4.3 4.3
4	39	♀	0.91	185/100	185/95	110/90	120/90	0.764 0.852	0.158 0.055	0.072 0.088	0.994 0.995	0.876 0.779	0.104 0.168	0.013 0.049	0.993 0.996	2.8 2.6	3.4 3.0	4.9 4.0

higher frequencies are damped more in the place where stenosis occurs.

v_1 is considerably lower than in healthy subjects, 2.6—4 m/sec which shows that the fundamental vibration is transmitted in another way than in the case of the latter. Compared with the "usual" hypertensive cases examined in this work, v_1 is still more obviously lower in the cases of isthmus stenosis.

No definite conclusions can be drawn for these cases from the magnitudes v_2 and v_3 . They are lower than the corresponding values in "usual" hypertensive cases. It is very probable that an extra phase lag is brought about by stenosis. In this respect the stenosis acts as though the aorta were extended at the place in question.

The So-Called Air-Chamber Theory and the Undulatory Theory.

In the so-called air-chamber theory, the heart is compared to a fire engine in which periodically repeated impulses force liquid out. The elastic vessels are dilated simultaneously and these exert pressure on the blood and continue to drive the blood onward when the heart is not pumping, *i. e.* during the diastole. The elastic arterial walls are considered to perform the same service as the air over the water in an air chamber (E. H. WEBER). In the undulatory theory, the physical laws for a wave movement are applied to the arterial pulse wave.

For purposes of quantitative analysis, the comparison of the elastic vessels to the air chamber in a fire engine is singularly inadequate. In an air chamber, the pressure is the same in all parts at approximately the same time. In his simple air-chamber theory, O. FRANK assumes that the pressure in the elastic vessels is the same (!). If we assume the existence of a pressure wave in an elastic tube system, the approximation is very rough. The theory by means of which FRANK and his school try to link the undulatory theory to the air-chamber theory is highly contradictory. On the one hand, they assume that the elastic vessels constitute an "air-chamber" with equal pressure in all parts; on the other, they assume when calculating the length of the air-chamber a pulse wave which, moreover, is reflected (WEZLER and BÖGER). Since we have to reckon with a pulse wave in the elastic vessels which is strongly reflected, the variations in pressure

in the different parts of the elastic vessels will likewise differ from one another. This fact has been well shown by HAMILTON and Dow in experiments on dogs. Both the systolic pressure and the blood pressure amplitude increase on the stretch aorta-femoral artery, while the mean pressure changes only negligibly. The latter is an expression of the small friction losses in the large vessels. The blood pressure amplitude may increase up to 90 %. It appears clearly from my investigations that the pulse wave is made up of a powerfully reflected wave. The fundamental vibration, which is decidedly dominant, is strongly reflected. In certain pathological cases the reflections are increased, in that even the first and possibly the second partial are reflected. Thus very probably the whole wave. It is clear that all attempts to calculate stroke volumes with the aid of the air-chamber theory come to grief. These stroke volume formulae usually contain the length of "the air chamber" and blood pressure amplitudes, which latter vary in different parts of the elastic vessels.

It appears from my investigations that with the aid of the undulatory theory one can obtain information not only about the nature of the pressure variations, but also about the transmission, of the pulse wave under physiological and pathological conditions.

It seems also to be possible to deduce a formula for the stroke volume from the *undulatory theory*.

Summary.

The author has made a study of the pulse wave in the aorta (subclavian artery-femoral artery). In a short critical-historical survey, an account is given of earlier studies of the pulse wave.

To record pulse curves, the author has used piezo-electric technique. The principles of this technique are described.

A mathematical-physical method of studying the nature and transmission of the pulse wave is given on the basis of *the undulatory theory*. The central and peripheral pulse curves are analyzed into simple sine waves with the aid of Fourier-analysis. A formula is given for the time mean value of the total elastic vibration energy per unit length of the vessel and a corresponding formula for each sine oscillation is also given. The pulse curves are characterized by indication of the different sine oscillations contained in the entire oscillation. Each individual sine oscillation on its way through the vessel is considered by itself. The velocity of an advancing sine wave in the aorta can be calculated with the help of the formula given from central (subclavian artery) and peripheral (femoral artery) pulse curves recorded simultaneously. This velocity is then independent of the frequency and on the whole independent of the measurement distance. For a reflected sine wave, on the other hand, one obtains with the formula given an apparent velocity which is different from that of an advancing wave and is dependent on both the measurement distance and amplitude conditions.

The results of the experiments can be summarized as follows:

1. The three first oscillations take up virtually the whole oscillation course in the pulse wave (from the point of view of energy). How large a part each of these oscillations takes has been calculated. The fundamental oscillation is the strongest.

2. The pulse wave is a strongly reflected wave. It has been shown experimentally that the fundamental oscillation is very strongly reflected, whereas the first and second partials are transmitted without any reflection to speak of. The speed of transmission of these oscillations (the first and second partials) is thus practically the same, and it increases with age as the

elasticity modulus of the wall of the aorta increases. The transmission of the pulse wave has been studied in 58 healthy persons.

3. A connection between the wave velocity, the course of the pressure and of the cardiac output is given under certain suppositions. This connection is deduced from *the undulatory theory*.

4. With the object of obtaining some general idea of the nature of the pulse wave in pathological cases, a small number of hypertensive patients have been examined. The size of the material does not permit systematization. The following deviations are to be noted:

a) The fundamental oscillation is more dominant than in healthy subjects.

b) The reflections are increased; both the fundamental oscillation and the first partial are reflected.

c) The velocity of an advancing wave is greater.

5. In cases of isthmus stenosis, the transmission conditions and damping of the pulse wave are changed as a result of constriction in the aorta. This fact has been shown experimentally, and is of interest not only from the hemodynamical but also from the clinical-diagnostic point of view.

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APPENDIX.

Technical Description of the Apparatus.

By *Rune Elmqvist.*

The apparatus that has been used in this investigation has been on the market¹ for several years but it has not yet been described in the literature. Therefore a more detailed technical description may be of interest.

The sphygmograph consists of a piezoelectric receptor, which is connected over an RC-network to the amplifier of an electrocardiograph.

Fig. 19 shows a cross section of the receptor. A piece of molded bakelite (1) supports the other parts. The Rochelle salt crystal element (2) is of the bender bimorph type. Its dimensions are about $18 \times 10 \times 0.8$ mm. One end of the crystal element is fastened to the bakelite base by a pair of rubber pieces in order to protect the element from breakage. The free end of the element is connected to a pelotte (3) by means of a metal wire (4). The pelotte is fixed to the metal housing (5) by a 1 mm. perforated brass disc (6), which has a free diameter of 30 mm. The cable from the receptor is a shielded microphone cable (7) connected to the electrodes of the crystal element.

The electrocardiograph is of the amplifier type for simultaneous recording of four leads. It has three battery-driven amplifiers and four oscillographs. For simultaneous recording of two pulse curves only two amplifiers and oscillographs are used. The amplifiers are of the RC type and consist of three stages each. In all stages battery pentodes DAF 11 are used. The tubes, however, are connected as triodes. The time constant of the amplifiers is 1.5 sec. In the anode circuit of each of the output stages is a high impedance electromagnetic oscillograph. Its resonant frequency is about 600 cycles, and it is a little underdamped. The

¹ Manufactured by Järnhs El. A.B. Stockholm.

deflections of the oscillograph mirrors are optically recorded on a continuously running silver bromide paper, 100 mm. wide. The speed of the paper is 40 or 100 mm/sec. The sensitivity of each channel may be independently varied, and it is about 25 mm/mV max.

The crystal element may, in the frequency range of interest here, be considered as a pure capacitance in series with an Emf.

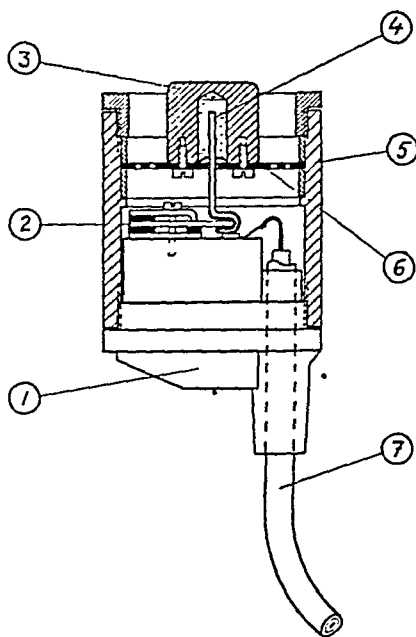


Fig. 19.

which varies proportionally to the pressure. The magnitude of the capacitance is about 1000 pF. In order to get a defined potential on the grid of the input tube, the grid resistor should be not higher than about 10 megohms. But if the receptor is connected to the input circuit with a shunt of 10 megaohms, the time constant will be of the order of only 0.01 sec., which corresponds to a low frequency limit of about 16 cycles. The low frequency limit for a sphygmograph should not, however, be higher than about 0.2 cycles. The time constant of the input circuit has therefore to be augmented. This augmentation of the time constant is accomplished by a condenser of 0.25 μ F, which is shunted over the receptor and the 10 megohm grid resistor. The time constant of the input circuit then is 2.5 sec. and the low frequency limit of the whole apparatus about 0.2 cycles.

The sensitivity of the sphygmograph varies a little with differ-

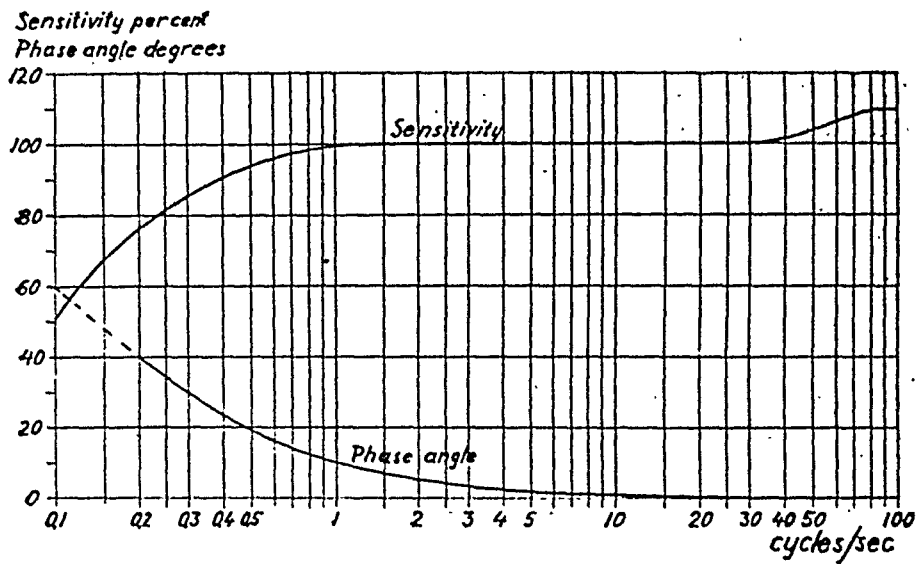


Fig. 20.

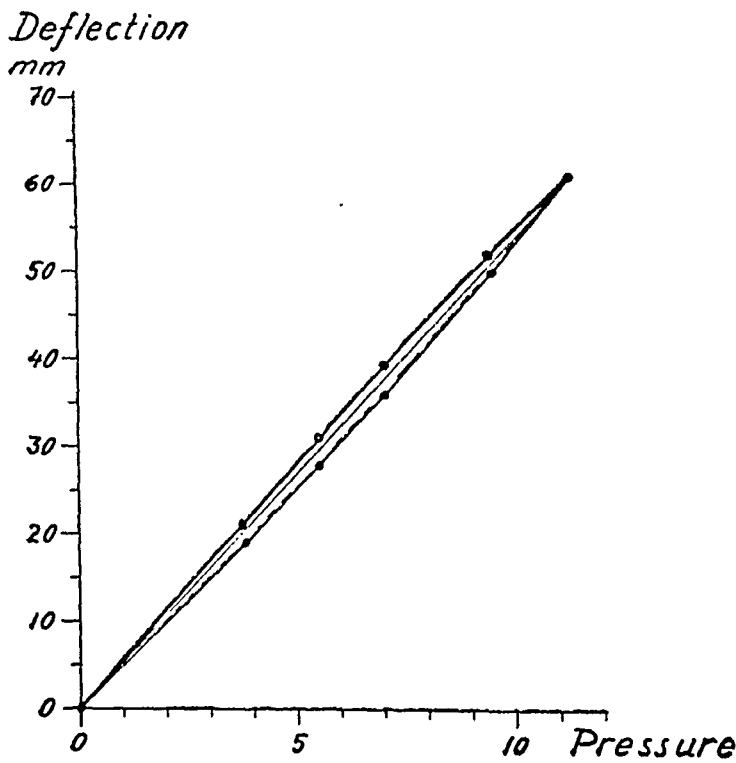


Fig. 21.

ent receptors, but that does not matter, because no absolute measurements are made. As an average, a pressure of 0.5 grams on the pelotte (or a displacement of about 0.01 mm) will give the grid of the input valve a potential of about 1 mV, which corresponds to a deflection of 25 mm max. This sensitivity is large enough even for recording from places where the pulsations are very weak. In most cases the amplification has to be lowered considerably in order to get traces of convenient size.

Fig. 20 shows the uniform response of the apparatus in the frequency range from 0.5 to 50 cycles. The reason for the slight increase of sensitivity between 50 and 100 cycles is that the receptor has its resonant frequency at about 100 cycles.

The current through the oscillograph is proportionate to the pressure within ± 30 mm. deflection. The oscillograph, however, unfortunately has a certain amount of hysteresis, which gives a slight degree of amplitude distortion. Fig. 21 shows the deflection as a function of the pressure on the pelotte. The right curve branch represents rising and the left one falling pressure. The straight line which would have been obtained if there had been no hysteresis is drawn between the two branches. The figure shows that the error due to hysteresis is about 5 per cent of the deflection.

ACTION POTENTIAL
AND
DIAMETER OF ISOLATED NERVE
FIBRES UNDER VARIOUS
CONDITIONS

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by

HELGE HERTZ

København

1947

*Denne Afhandling er af det lægevidenskabelige Fakultet antaget
til offentligt at forsvares for den medicinske Doktorgrad*

København 21. Oktober 1946

*Knud O. Møller
pro. dec.*

Dedicated to

Valborg and Peter Hertz

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P R E F A C E

THIS work was prepared and commenced in the Neurophysiological Institute in Copenhagen in the years 1940—43. Most of the experiments were carried out in the Physiological Institute of the University of Lund, Sweden, (1943—44), and finally the work was finished in England (1944—45).

I am deeply indebted to Fritz Buchthal, M.D., for electro-physiological education and friendly constructive criticism throughout. I wish to express my gratitude to Prof. Mogens Fog for support when the work was first planned, to Prof. August Krogh for very valuable discussions about permeability problems, and to Prof. Emanuel Hansen, Prof. Einar Lundsgaard and Prof. George Kahlson for permission to work in their laboratories. I am also indebted to Civil-Engineer Paul Binzer, to Edmund Kaiser and Georg Knappeis for technical advice, and to Dr. William A. Cobb, London, for correction of the English manuscript.

Last, but not least, it is my wish here to acknowledge the personal help and moral support received from the entire staff of the three institutions and from my private friends in Denmark, Sweden and England — without those encouragements it would have been impossible to finish this work under the somewhat difficult conditions.

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INTRODUCTION

AMONGST methods in use for the purpose of studying the nerve impulse, the recording of action potentials occupies a unique position. Firstly, it is possible to measure the electrical changes quantitatively; secondly, the investigations of *Hermann* (1899) have shown that the action potential is an essential part of the nerve impulse, perhaps the impulse itself, a view which has been even better established through the work of *Hodgkin* (1937); thirdly, it has become easier to measure action potentials by the use of modern electrical equipment, such as the valve amplifier and the cathode ray oscillograph. In view of these facts it is natural to use the action potential as an indicator for the nerve impulse when the effect of various chemical influences upon nerve has to be investigated.

Adrian (1928 and 1931) has made it clear that it is necessary to work on isolated muscle- and nerve-fibres when the more intimate function of muscle or nerve elements is under consideration, to prevent the interference of other elements and the influence of non-active tissue, blood vessels, etc. Therefore, we have made our experiments on isolated fibres, or preparations which are physiologically equal to isolated fibres. To demonstrate which factors are acting when the impulse is travelling down a nerve fibre we investigated the action potential under various conditions, especially with the isolated fibre immersed in solutions containing different amounts of ions.

Several authors have tried the effect upon the nerve of solutions with various electrolyte content: — *MacDonald* (1900—1902), *Höber and Strohe* (1929), *Cowan* (1934), *Fenn* (1934), and *Helen Treadway-Graham* (1933). They have shown that potassium is liberated from the nerve during the impulse, and that potassium ions reversibly decrease the action — and demarcation — potentials, and, moreover, that this potassium effect is antagonistically

influenced by an increase of calcium ions. *Cole and Curtis* (1938, 1939) have shown that the impedance of a cephalopode nerve fibre decreases concurrently with the increasing negative action potential, and conversely. *Webb and Young* (1940) have tried to correlate the amplitude of the action potential with the diffusion potential due to the difference in potassium content inside and outside the interface surrounding the axis cylinder. *Lehmann* (1937 a, b, c) has examined the effect of anoxia, pH changes, and decrease of calcium ion concentration on the after-potentials of whole mammalian nerves.

For several reasons we thought it useful to explore these problems once more. Firstly, many previous investigations have been made on whole nerves, which is likely to cause confusion (*Adrian*, see above); secondly, modern electrical technique has made it possible to obtain a true record of the development of the potential according to time, which was not obtainable by earlier investigators who worked with moving-coil galvanometers; thirdly, in all experiments it has been necessary to use electrical stimulation, as a result of which several earlier records, as for example *Lehmann's*, were disturbed by stimulus escape. No previous authors have calculated the variable shunt effect of the fluid between the leading-off electrodes, which of course influences the records considerably, even when the fluid is only a water film on the surface of the nerve or fibre. Different techniques have been used in all the investigations mentioned, for which reason it is difficult to correlate the results given by one author with those provided by another. *Erlanger and Gasser* (1937) have compared the action potentials from different nerves under normal physiological conditions with the diameters of the fibres, but no description has been found in the literature of an attempt to use the variations in diameter of one single nerve fibre immersed in different solutions as an indicator of the permeability of the membrane, or to correlate these variations with changes in the action potentials from the same fibre, recorded at the same time. Such measurements of the diameters of single cells have been used with good results as an indicator of the water permeability of red blood cells and of muscle fibres, and we therefore thought it useful to apply the method to nerve fibres.

CHAPTER I

SHORT HISTORICAL SURVEY

Galvani in 1786 demonstrated muscular twitchings in the legs of a frog when the muscles or their nerves were touched by both ends of metal bow, and *Volta* supplemented *Galvani's* observations, but the Italian physiologist *Matteucci* made the first critical analysis of the whole subject, with particular attention to the nerves. In his classical work of 1840 "*Essai sur les Phénomènes électriques des Animaux*", and in some articles, most of which appeared in his own periodical "*Il nuovo cimento*", he arrived at two conclusions: — (1) that the nerve impulse is due to "a condition of altered excitability travelling down the nerve"; and (2) that electricity is really generated in muscles and nerves during their activity. He described a method to demonstrate this in the absence of sensitive electrical instruments: he placed the nerve of a nerve-muscle preparation on the muscle of another the second nerve in this way being stimulated by the action potential of the first muscle. He propounded the theory of core-conduction, which featured prominently in the discussion of the nerve impulse during the remaining part of the 19th century.

Du Bois-Reymond reported in his "*Untersuchungen über thierische Elektrizität*" (1848—1849) and "*Muskel- und Nerven Physik*" (1875—1877) a considerable amount of experimental work. He succeeded in measuring the electrical phenomena of nerves and muscles by a purely electrical instrument, a polarised needle galvanometer, the so-called multiplier, which was constructed for the purpose. He invented the non-polarisable electrode to prevent galvanic effects from interfering with the measurements, when the metal conductor touched the tissue. By using these instruments *Du Bois-Reymond* was the first to differentiate between the demar-

cation potential led off from an injured part of a tissue to an uninjured part, and an action potential which could be recorded from muscles and nerves when they were stimulated electrically, or in some other way. He showed that the excited part of a tissue, as well as the injured part, is always negative to the intact.

In collaboration with his pupil, *Hermann*, he formulated the so-called molecular theory. *Hermann* (1905) stated that it was impossible, with the most sensitive galvanometers of that time, to record any potential difference between uninjured parts of resting tissues. He made calculations (1872) upon *Matteucci's* core-conduction theory, and constructed a core-conduction model. *Hermann's* investigations (1898), later elaborated by *Pflüger*, (1899), of the alterations in the irritability of the nerve when a constant current was passed through it — the so-called physiological electrotonus — has been, and still is of great importance for all theories concerning the mechanism of nerve conduction.

With the works of *Gibbs* (1876—1878) and *Helmholtz* (1876) it became possible to consider the origin of electrical forces from a more quantitative point of view. This was the basis upon which *Nernst* (1899), *Bernstein* (1902), *Haber* and *Klemensiewicz* (1909), built up their theories of biological potentials as being membrane- and concentration-chain-potentials. From the beginning of this century the investigations were conducted along several different lines. *Haber* (1909), *Bernstein*, (1912), *Beutner*, (1920) and others continued to work on nerve models of the concentration-chain type.

All this work was based upon the theory of the cell being surrounded by a passive semi-permeable membrane maintaining a concentration difference between extra cellular and intra cellular fluids, which concentration difference was supposed to account for the electrical potentials. *Hevesy* (1937) and later *Krogh* (1938) have shown by means of radioactive isotopes, that living membranes probably have an active selectivity for certain ions in preference to others, a view, which was confirmed by *Lundegårdh* (1940) on vegetable membranes by the investigation of surface potentials.

While the work mentioned was mainly concerned with finding the source of the electrical potentials measured in the nerve, the

action potentials were meanwhile used by others as an indicator of nervous activity. Action potentials were measured first by means of a capillary electrometer, later by *Einthoven's* string galvanometer, and in recent years by amplifiers and various oscillographs. The physiological activity of the various fibres in a nerve trunk was investigated in this way by *Adrian*, (1931), *Matthews*, (1929), and *Erlanger and Gasser*, (1937), while potentials were recorded from single fibres, in order to clarify the mechanism of nervous conduction *Erlanger and Blair*, (1934). *Blair and Erlanger*, (1933), *Kato*, (1934), and *Hodgkin*, (1938), devised techniques for work, on isolated fibres. In recent years, the giant fibres from cephalopodes have been used *Young*, (1937), *Hodgkin*, (1939), and others. — *A. V. Hill*, (1935), *Lapique*, (1937), *Monnier*, (1934), and later *Katz*, (1940), have studied the theory of electric excitation of the nerve. The results of *Hill's* investigations have been given a further mathematical treatment by *Rushton*, (1937), and also *Rashevsky*, (1933), contributed to a mathematical theory of nerve excitation.

The important work by *Loewi*, (1921, 1922) and by *Dale*, (1938), and his collaborators on chemical transmissions in the neural and neuro-muscular connections has led to experiments with chemical transmitters in the peripheral nerve (*v. Muralt*, (1939), *Nachmansohn*, (1941)). It has been shown by *v. Muralt*, (1942), that "acetylcholine-equivalents" are present in higher concentrations in the excited nerve than in the not excited one, but no final proof has yet been given that chemical transmission is an essential part of the conduction of impulse in the peripheral nerve.

The idea of measuring the electrical potentials in the nerve during alteration of the surrounding chemical conditions was first introduced in 1900 by *MacDonald*, who investigated the dependence of injury potentials upon the fluid which surrounded the nerve. His experiments were original, but his electrical instruments were inferior by modern standards, and he was not concerned with, whether the nerves were dead or alive. In part of his work he even preferred to use dead nerves in trying to answer what was, at that time, one of the most important physiological questions, whether the observed electrical potentials were due to vital forces or were

purely physical phenomena. *MacDonald* showed for the first time, that an increase in potassium content in the surrounding fluid decreased the demarcation potential of the nerve. In 1929 *Rosenberg* and *Kitayama* showed that increase of potassium also decreased the action potential. In the same year it was demonstrated by *Furusawa* that the potassium concentration is much higher inside the nerve than it is in the normal intercellular fluid. In 1934 *MacDonald's* observations were tested on living nerves by *Cowan*, who investigated both the demarcation and action potentials under varied conditions. The recording of action potentials was used here only to indicate that the nerve was still alive, and the method of recording, he used a moving coil galvanometer, prevented him from registering any details of the action potential. The most important part of *Cowan's* work was the analysis of the ionic content of the nerve and of the intercellular fluid, and especially the alterations in the ionic content of the fluid, surrounding the nerve during the experiments. In this way he demonstrated the important fact that the nerve during its activity loses potassium ions to the fluid round it. *Cowan* also showed that the action potentials decrease when rubidium or caesium ions are added to the surrounding fluid, as was the case with potassium ions, but that the potassium effect on the demarcation potential could not be provoked by caesium or rubidium ions. The potassium effect could be partly counteracted by calcium, and all the effects described were instantaneous and fully reversible. Hypertonic solutions increased the demarcation potential. Nerves which were asphyxiated could be resuscitated by oxygen, but if the asphyxia was of long duration, they had to be washed in sea-water (the experiments were made on crabs) to resuscitate them. *Cowan* concluded that this was due to potassium loss from the nerves during anoxia. Finally, in some experiments on the non-medullated nerve of the *Maia Squinado*, he measured the injury potential and the length of time, for which it could be maintained. It was shown that, with a potential of 30 millivolts, a current of about 2×10^{-7} amperes could be obtained during an hour or two, and he concluded that this current was transported through the nerve membrane by potassium ions, but like earlier investigators, he did not take into account the effect of the water film round the nerve during the experiments.

In 1932 it had been shown by *Bishop*, and in 1933 by *Helen Treadway-Graham* that potassium could decrease the potentials from the nerve, and that this effect was antagonistically influenced by calcium. *Erlanger* and *Blair* had in 1932 compared this action with cathodal and anodal polarisation. None of these investigations are as conclusive as those by *Cowan* in 1934. He showed that the potassium content of nerves varied between 3.02 and 4.8 mg per cent (0.78—1.23 millimolar K^+), and that potassium apparently did not diffuse from the nerve to an isotonic glucose solution. The permeability of the nerve membrane, especially to potassium, was also investigated by *Höber and Strohe*, (1929), and the permeability to Ca^{++} by *Tipton*, (1934). However, in spite of all these experiments the permeability problem of the nerve fibre has not been investigated as thoroughly as is the case with the muscle membrane, especially after the properties of the latter were explored by means of radioactive isotopes.

In 1937 it was shown by *Lehmann*, that rhythmic spontaneous discharges are provoked in nerves which are treated with citrate. These changes can be provoked or speeded up, if the pH is raised to about 8 or if the nerve is asphyxiated; they can be counteracted by an increase of potassium. In contrast it was reported in 1939 by *Brown and MacIntosh* that the injection into the carotid artery of a cat of 2—5 mg of potassium chloride in isotonic solution provoked discharges from the sympathetic and vagus nerves in the neck; these discharges could be counteracted by double the amount of calcium chloride. They are probably due to stimulation of the ganglionic cells, a theory which would also explain the sympathetic stimulating effect described, and used therapeutically, of injecting potassium chloride into the cisterna magna.

CHAPTER 2

TECHNIQUE

The technique used in this work can be divided into three main parts: — (1) *Preparation and Micro-Technique*: dissection of the nerve-muscle preparation, mounting of it in a moist chamber, microdissection of the single nerve fibre, and microscopical recordings of the diameter of the fibre. (2) *Electrical and Photographic Technique*: the source of stimulation, electrodes, leading-off electrodes, the amplifier and cathode-ray oscillograph, with the synchronising device to make the sweep of the cathode ray simultaneous with the excitation impulse, and the bridge arrangement to control the resistance between the leading-off electrodes. Photographic recording from the oscillograph screen and the analysis of the curve will also be mentioned in this section. (3) *Preparation of solutions and standardisation of their electrolyte content, pH, oxygen-carbon dioxide tension, and temperature.*

I. *Preparation and Micro-Technique.*

Isolated nerve fibres from the sciatic nerve of the frog (*Rana temporaria* vel *esculenta*) have been used in this work. Nerves from frogs are good to handle on account of their temperature indifference, and easy obtainability. The giant nerve fibres of the cephalopode are better to dissect and give bigger action potentials; furthermore it is possible in the cephalopode fibres to estimate almost exactly the absolute amplitude of the action potentials by use of an inner electrode, as described by *Hodgkin and Huxley* (1939). External events made investigations on the cephalopode nerve fibres impossible, but it is our hope to be able to do some control experiments on these fibres. The same species (*Loligo*) as

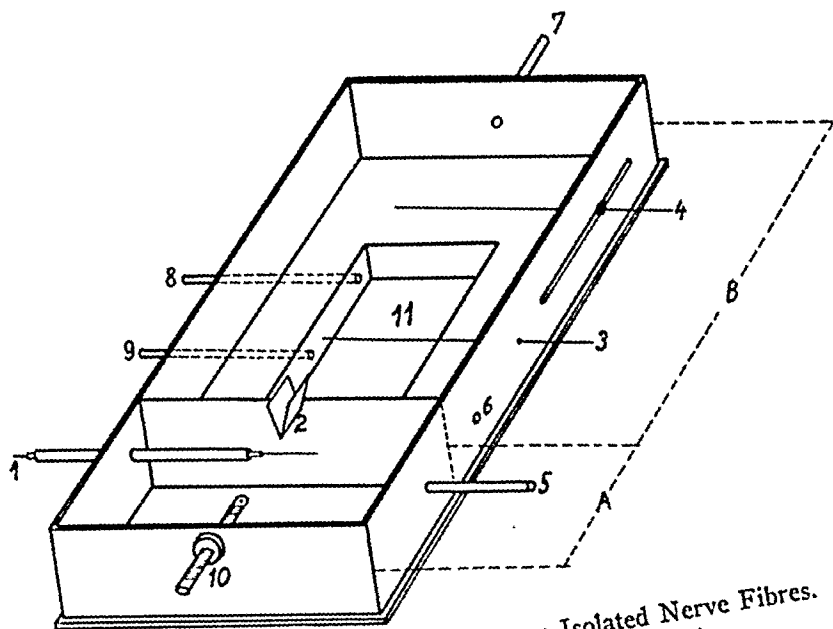


Fig. 1. Chamber for Experiments on Isolated Nerve Fibres.
A. Excitation part. B. Leading-off part.

- 1) Concentric shielded platinum excitation electrode.
- 2) Earthed trough-shaped platinum excitation electrode.
- 3) Earthed Ag-AgCl leading-off electrode.
- 4) Non-earthed Ag-AgCl leading-off electrode.
- 5) Inlet for oxygen or hydrogen to excitation chamber.
- 6) Outlet for oxygen or hydrogen from leading-off chamber.
- 7) Inlet for oxygen or hydrogen to leading-off chamber.
- 8) Inlet for water into the double floor for temperature regulation.
- 9) Outlet for water for temperature regulation.
- 10) Screw to adjust the tension on the nerve.
- 11) Glass double floor.

that used by *Young, Hodgkin, Huxley and Webb* can be caught in Danish waters.

Our preparation was made from a usual nerve-muscle preparation (sciatico-gastrocnemius). The muscle was put into the chamber shown in Fig. 1; a piece of silk was tied round the end of the nerve, and by this it was fixed to the ebonite screw (10). The part of the nerve just central to its attachment to the muscle was placed under the second leading-off electrode (4) and in contact with it. The nerve was thus resting on the first leading-off electrode (3), and the two stimulating electrodes (2) and (1). Slight tension was now applied to the nerve by tightening the screw (10), while the electrode (4) fixed it at the other end. By this method it was possi-

ble to control very accurately the tension of the nerve throughout the microdissection. The chamber was placed on the table of the dissection microscope with a magnification of 32 x, light was concentrated from beneath through the glass floor in the chamber (11), while another lamp illuminated from above the part of the nerve being dissected. With a sharply pointed knife the nerve sheath was incised longitudinally, and, on account of different elasticities, it was then a simple matter to part the fibre bundle and the connective tissue with a needle, and cut across the whole sheath with *Buchthal's* scissors. After this had been done, the screw (10) was loosened a little to ensure that the tension on the nerve fibres was not too heavy. Bundles of fibres could then be isolated, and after a suitable bundle of undamaged central fibres had been found, some of them were cut with the scissors. During this procedure it was observed, whether this was a bundle of motor nerve fibres by watching the twitchings in the muscle. If this was the case, the bundle was isolated with a needle, while other bundles were cut, except for one in the periphery of the nerve, which was not severed in order that it might take the tension. In the central bundle, fibre after fibre was now picked up with a pointed needle, either of stainless steel or of glass, and cut, until one large intact fibre was left. Now the nerve was completely slackened, and finally the peripheral bundle was cut. (Fig. 2).

Throughout the whole dissection the nerve was kept immersed in oxygenated Ringer's solution (see later), and occasionally the action potentials were recorded for control. Sometimes the fibre was imperceptibly damaged, or a sensory (proprioceptive) fibre was isolated, in which case a suitable action potential could not be obtained, and it was necessary to make an entirely new preparation. Attempts to test the irritability of the isolated fibre by means of galvanic forceps (*Kato*), or a concentric needle electrode, invariably damaged the fibres, and these methods were abandoned. When the preparation was not to be used for recording the diameter of the fibre, but only for measuring action potentials, the dissection was not carried to anatomical isolation of one single fibre, but was finished off when the preparation gave an "all or none" response, when the stimulus was increased. (cf. *Blair and Erlanger*, 1933, page 527). When the micro-dissection was finished, the prepara-

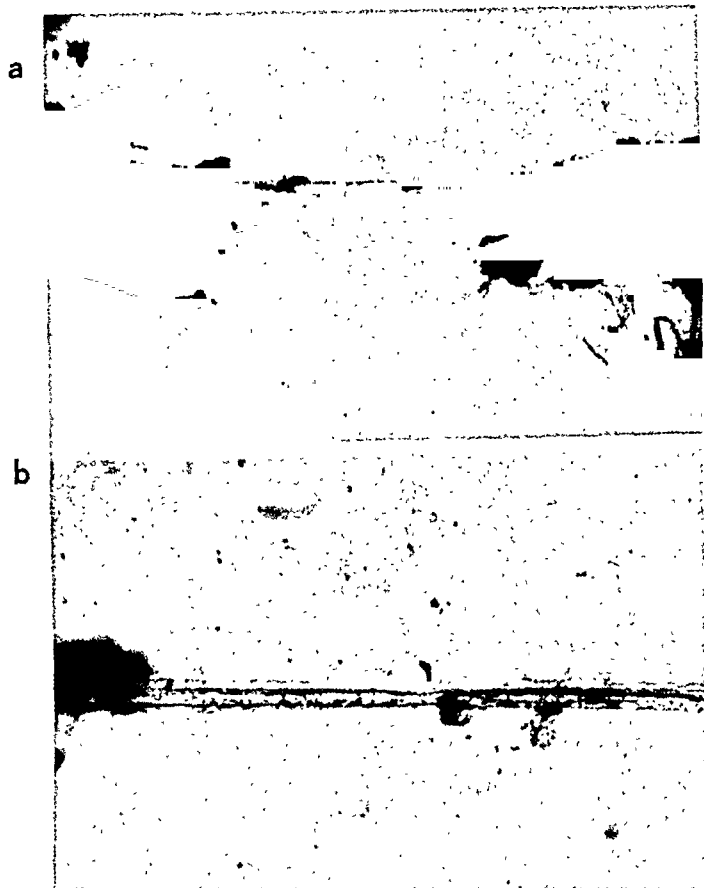


Fig. 2. Isolated Nerve Fibre.

- a) Magnification: $15\times$.
- b) Magnification: $150\times$.

tion was made monophasic by crushing the nerve between the leading-off electrodes with a pair of forceps. If one single fibre was isolated for measuring variations in diameter, a small piece of finely powdered graphite was put on the fibre by sucking the surrounding fluid away from it, so that the graphite attached itself to the fibre by capillary attraction, and made it possible to measure the diameter of the nerve at exactly the same spot throughout the experiment. The different fluids were applied to the nerve fibre through a finely pointed pipette. The whole chamber was placed slightly sloping towards "the stimulation end" so that superfluous fluid ran down into the relatively deep portion. For the purpose of measuring diameters the dissecting microscope was replaced by

an ordinary microscope, fitted with a Zeiss apochromatic water-immersion objective, with a magnification of 70 x and an aperture of 1.25. A Zeiss micrometer with movable cobweb was used in the eyepiece. The system was calibrated against a stage micrometer. The diameter of fibres measured, varied between 4.46μ — 12.05μ under physiological conditions. Before the actual experiment started, the diameter of each nerve fibre was measured ten times to ensure reliability of the results.

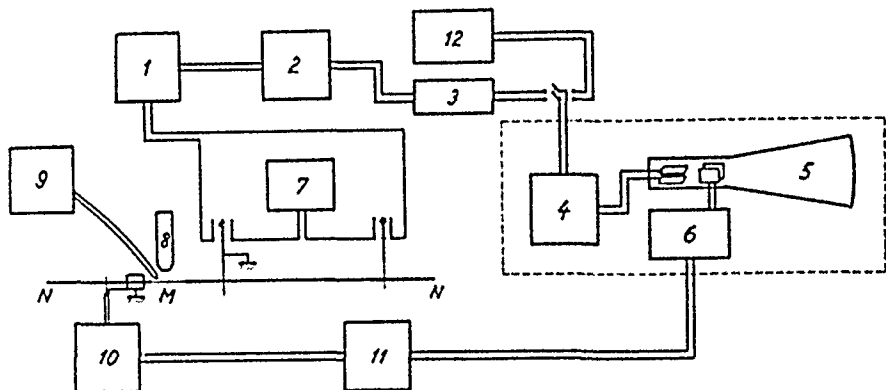
These measurements from a series of experiments were subjected to statistical analysis, and gave a standard deviation:

$$\sqrt{\frac{\sum d^2}{n-1}} = 8.65 \text{ per cent,}$$

$$\text{and a mean error of: } \sqrt{\frac{\sum d^2}{n(n-1)}} = \pm 1 \text{ per cent.}$$

II. *Electrical and Photographic Technique.*

The nerve was stimulated with brief condenser discharges (Fig. 3). Brevity was secured by a gas discharge tube, the extinction voltage being that, at which the discharge was cut off. The condenser discharge were operated by a relay (Fig. 4), with various condensers and resistors to eliminate interference from it. The main condenser, was charged through the relay from a 100-volt battery. To prevent stimulus escape the entire apparatus was built in a metal box, the relay being in one completely separate part of the box, the relay contact and the condensers in another, and finally the battery in a third. The stimulation current was conducted to the excitation chamber [Fig. 1 A] through a concentric shielded cable earthed with the outer metal box and the inactive stimulation electrode. Round the stimulating platinum electrodes was built a brass chamber, as shown in Fig. 1. The active electrode, which was connected to the central lead in the cable, was in the centre of this chamber, while the non-active one was soldered to the edge of the chamber and earthed together with it. To prevent stimulus escape it was important, that the earthed non-active stimulating electrode was trough-shaped and that it was at least six millimeters long. Only in this way was it possible to secure adequate contact between the earthed electrode and the nerve, and



- 1) Amplifier first stage.
- 2) Amplifier second stage.
- 3) Low pass filter.
- 4) Amplifier third and fourth stage.
- 5) Cathode ray tube.
- 6) Sweep oscillator.
- 7) Bridge arrangement to measure a.c. resistance between electrodes.
- 8) Microscope to measure diameter of fibre.
- 9) Galvanometer with thermo-couple to measure temperature.
- 10) Stimulator.
- 11) Relais mechanism to synchronise sweep and stimulation.
- 12) Time base laboratory generator.

Fig. 4. Stimulator. The 0.5 μ F condenser is discharged by the relays through a gas discharge tube. Various resistors and condensers prevent stimulus escape and disturbances from the relays.

thereby prevent stray currents from the excitation discharge from escaping down the moist outer surface of the nerve, giving rise to disturbances. It was also necessary to earth the leading-off electrode nearest to the stimulated part of the nerve, even when a

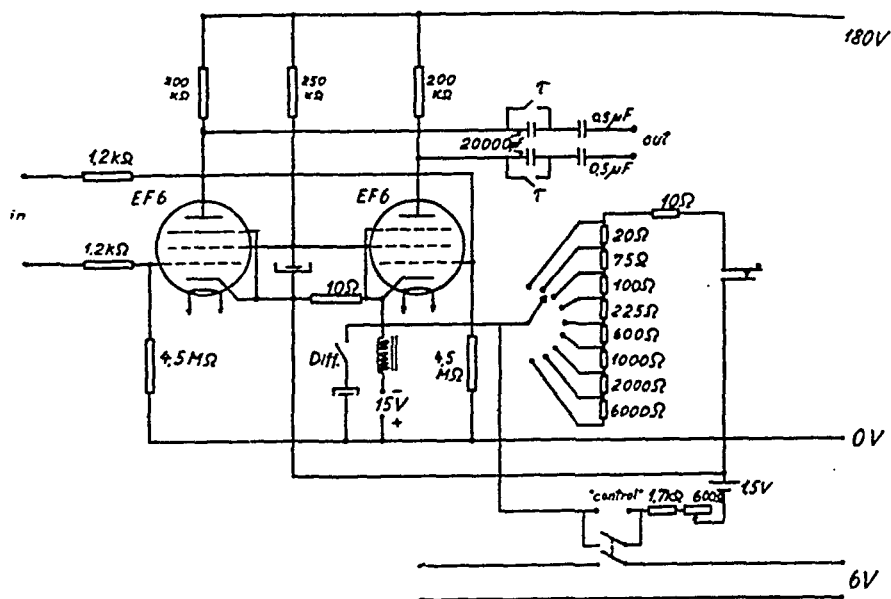


Fig. 5. Amplifier. Input stage. As described by *Buchthal and Kaiser*, and provided with a calibrating arrangement.

differential coupled amplifier was used. The action potential was led off by two Ag—AgCl electrodes, one earthed as described, and both led through shielded cables to the amplifier grids.

The amplifier consisted of four stages, of which the first two, in separate boxes, were battery driven. The first stage was [Fig. 3, 1] differential coupled, modified from a differential amplifier designed for electro-encephalography by *Buchthal and Kaiser* (1943) (see Fig. 5 for circuit diagram). Two Philips' EF 6 tubes heated with current from a 6-volt storage battery were used. A calibrating arrangement with 1.5-volt battery, various resistances, a switch and a push button contact permitted the introduction of known EMFs from 2.5 microvolts to 5 millivolts, the circuit [Fig. 5] permitted this to be done without disturbances in the amplifier lead. This stage, together with the calibrator and 180-volt anode battery, were enclosed in a metal box which was unearthed,

as earthing of it gave electrostatic disturbances. The battery voltage could be measured from outside the box.

The second stage was a Philips' "pre-amplifier" [Fig. 3, 2] to the cathode ray oscillograph, of the type V with anode and filament batteries built in, and using one EF 6 valve. Between this and the third stage a low-pass filter [Fig. 3, 3], cutting off sharply at about 2000 cycles, excluded high frequency disturbances. This filter was only used, when working with a rotating converter, which produced high frequency disturbances. The two last stages were push-pull coupled [Fig. 3, 4], built, together with a cathode ray tube and its connections, in the Philips' unit, and like the cathode ray tube, driven from the mains. The input of the two last amplifier stages contained in Philips' unit GM 3156 was altered to differential input.

The time-base was provided by introducing a 1000/sec. A. C. from a Philips' laboratory generator [Fig. 3, 12], [type GM 2305] directly into the third stage. Sometimes 500/sec. or 2000/sec. were used, but this is always mentioned in the text. By this arrange-

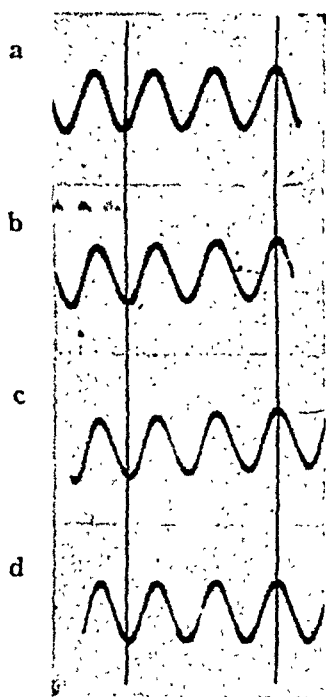


Fig. 6. Checking of Time Base Constancy. a, b, c and d is a 1000 AC recorded 0, 1, 2 and 4 hours after the experiments started.

ment, with only a single-beam tube, it was not possible to time-base simultaneously with the actual record, but the constancy of the generator and the sweep mechanism was checked by taking photographs of the time-base before, during and after an experiment, as illustrated in Fig. 6.

In order to ensure constant resistance between the electrodes, the leads from the preparation could be switched over from the input of the first amplifier stage to the input of a bridge unit, a Philips' Philoscope, type GM 4140 [Fig. 3, 7], by means of which it was possible to measure the resistance in the electrode lead, the nerve and the fluid round the nerve. The impedance was measured at a frequency of 1000 cycles per/sec., provided by the time base generator, as this was considered to be a higher frequency than any part of the action potential, and prevented polarisation. The main object in this arrangement was to control the resistance in the fluid surrounding the nerve preparation and ensure that this resistance was maintained constant throughout the experiment and particularly also when the fluid had been changed. This was considered to be of great importance, as it is possible to obtain varying amplitudes of the action potential simply by altering the shunt effect of the fluid. To protect the nerve against excessive current during the resistance measurement a 50,000 ohms resistor was put in the lead to the bridge, and it was important to make the duration of each measurement as short as possible. Corrections were made for this resistance.

The cathode ray oscillograph was arranged in such a way that single sweeps could be obtained. The sweep mechanism in the oscillograph Philips' GM 3156 is so constructed that the spot is fixed on the right hand side of the screen with a voltage of about 45 volt applied to the synchronisation terminals. If this voltage is momentarily reduced to about 0, the spot will jump to the left hand side of the screen, and there begin a single sweep, after which the spot stops in its original position. In our apparatus the voltage was momentarily reduced by short-circuiting the synchronisation terminals through a condenser [Fig. 7]. Various condensers and resistances were introduced to eliminate artifacts and to obtain a suitable delay of the stimulus relay. By altering the attraction time of the relay, the synchronisation between the single

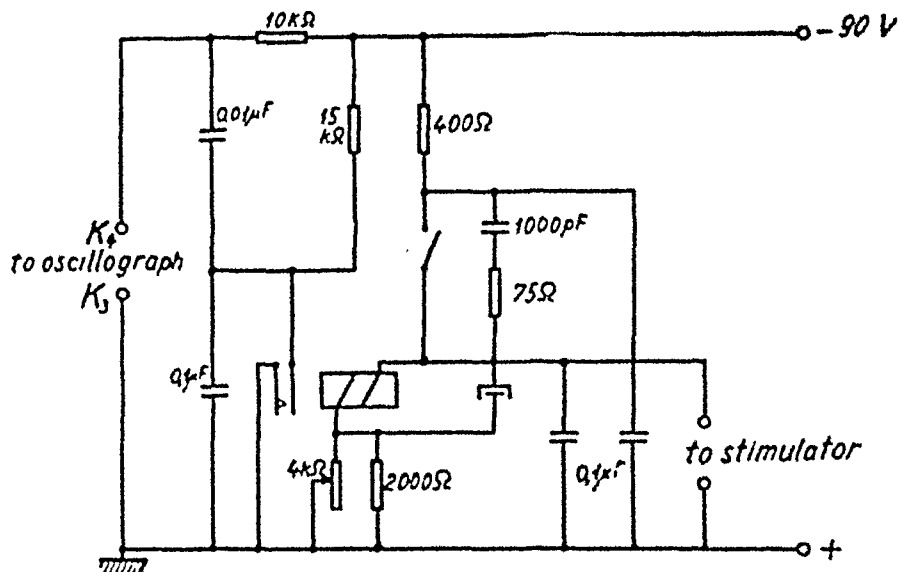


Fig. 7. Sweep-Stimulator Synchronisation Arrangement.

A full account of this arrangement is given in the text.

sweep and the condenser discharge in the excitation lead could be speeded up or delayed by some milliseconds, making it possible to bring any part of the action potential to the centre of the screen, even with different sweep speeds.

Between one X-plate of the cathode ray tube and earth, a 90-volt battery was placed to bring the beam outside the screen when it was stationary. This arrangement had two purposes: firstly it protected the screen, and secondly it prevented false light on the photograph from the stationary beam. Fig. 8 shows an example of how much stimulus escape and disturbance from the relay arrangement actually occurred with the usual amplifications, when a cotton thread of the same resistance as the nerve was placed

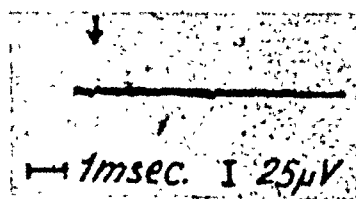


Fig. 8. Stimulus Escape

A wet cotton thread is placed between the leading off electrodes and the excitation electrodes. The arrow indicates the stimulus escape.

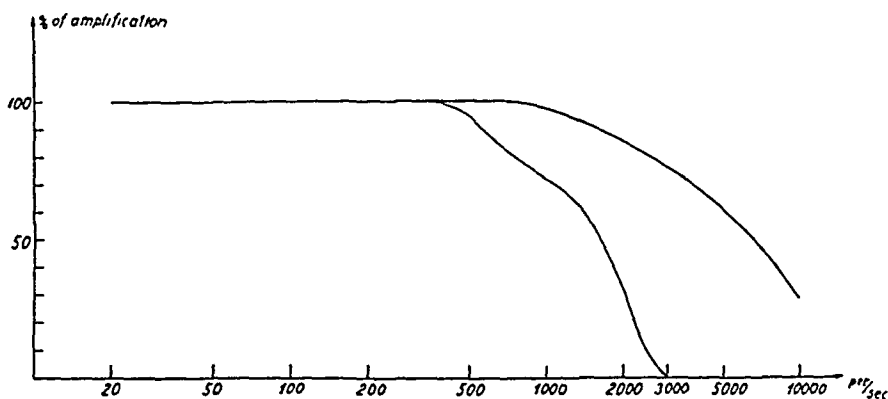


Fig. 9. Frequency Response
with and without a low pass filter as described.
Ordinate: Per cent of full amplification.
Abscissa: Frequency in cycles per sec.

between the electrodes, and Fig. 9 is a frequency response of the amplifier-oscillograph system with and without a filter as described.

The photographs of the transient trace on the screen were taken with a Leica camera. A summar objective $f=2$ was used, but was usually stopped down to $f=3.5$. An extension tube was fixed between the camera and the objective so that the focal distance of the objective was brought down to about 30 cm, and the camera was fixed up on the end of a black tube of that length and the same diameter as the screen, fitted to the oscillograph by a hinge, so that the actual recording could be made in full daylight; on the other hand it was possible to turn up the camera and observe the trace on the screen. Agfa Fluorapid film, which is produced for photographing X-ray screening pictures, was used as negative material, and with a tube with a blue fluorescent screen, it was possible to get satisfactory photographs even of very fast sweeps. Developing was done in ordinary metol-hydroquinone developer. The curves were magnified in a photographic enlarger and projected on to millimeter paper on which the outlines were drawn at approximately $4\times$ magnification, and measured.

III. Chemical Technique.

The Ringer's solution used contained 6.7 g NaCl, 0.2 g KCl, 0.11 g anhydrous CaCl_2 , and 0.2 g glucose per litre. Immediately before use a suitable amount of 5 per cent NaHCO_3 solution was

added to every 50 ml of the Ringer's fluid, and the resultant solution was saturated for 5—10 minutes with a mixture of 1 per cent CO_2 and 99 per cent O_2 . The amount of NaHCO_3 added was chosen in such a way that the Ringer's solution maintained for several hours a pH of 7.2 to 7.4 regularly checked with a glass electrode, which was adjusted against a *Sørensen's* buffer solution.

There was some doubt as to the optimal calcium content in the solution, for it is obvious from the literature that there is great difference of opinion on this matter. Despite the accurate analyses of calcium content of the blood and intercellular fluid [e. g. *Fenn* [1934]], it is by no means certain what proportion is ionised. The table on Fig. 10 shows the different values of calcium for artificial tissue fluids used by various authors, compared with the analyses of blood and plasma. In a work of this kind it is, of course, of minor importance which value is used, as the interesting point is the proportion between potassium and calcium, which was controlled throughout the experiments. The value 0.11 g anhydrous CaCl_2 in one thousand ml of water was chosen in accordance with figures given by *Krogh* [personal communication]. In making solutions it is important to remember that it is difficult to weigh CaCl_2 on account of its hygroscopic nature. After some trials it was found most satisfactory to use Merck's preparation of $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$, as this, when weighed quickly, has an almost constant weight. The actual CaCl_2 content was then ascertained by estimating the Cl^- content in the stock CaCl_2 solution by *Lehmann's* method [1944], and this stock solution was then used for preparing the final Ringer's solution. *Lehmann* uses an electrometric titration. He measures the electric charge of a silver electrode immersed into the test solution. When a silver nitrate solution is added from a micro-burette and the Cl^- in the test sample is precipitated, the potential falls very slowly at first, but later very quickly, and the curve is steepest at the point where the chloride is precipitated. It is possible in this way to estimate the chloride in the solution with great accuracy. Fig. 11 shows the apparatus used, and in the text beneath the exact procedure is described, all of which is taken from *Lehmann's* paper on the subject.

- 1) *Fenn's* Analysis of Frog plasma (*Fenn*: Electrolytes in muscle. *Physiol. Rev.* 16 1936).
 $K\ 2.5\ \text{mM/kg} = \text{ca. } 2.39\ \text{mM/l}$ $Ca\ 2.0\ \text{mM/kg} = 1.91\ \text{mM/l}$
- 2) *Fenn's* "Ringer" solution.
 $K\ 1.3\ \text{mM/kg} \frac{100}{75}$ $Ca\ 1.8\ \text{mM/kg} \frac{200}{111}$
- 3) Ours:
 $K\ 2.66\ \text{mM/kg} \frac{200}{75}$ $Ca\ 1.8\ \text{mM/l} \frac{200}{111}$ (0.69 mM/l med 6 H₂O)
- 4) *Barkan's* (*Barkan, Brömser and Hahn*) (*Z. Biol.* 74, 9).
 $K\ 1.33\ \text{mM/l KCl} \frac{100}{75}$ $Ca\ 1.18\ \text{mM/l} \frac{200}{111}$
- 5) *Boyle and Conway* (Potassium Accumulation in Muscle and Associated Changes *J. Physiol.* 100: 3-4, 1941).
 $K\ 2.5\ \text{mM/l}$ corresponding to 188 mg KCl (phosphate) $Ca\ 0.9\ \text{mM/l}$ (10 per cent Ca(C₆H₁₁O₇), H₂O).
 $2\ \text{ml to } 500\ \text{ml} \frac{448.39}{40.08} = \frac{1}{11.2}$
 $\frac{0.4}{11.2} = 35.6\ \text{mg}; \frac{35.6}{6} = 39.6\ \text{mg}$
- 6) *Gerlach* (*Biochem. Zeitschrift* 61: 125 1914)
 $K\ \text{---}$ $Ca\ 4.5\ \text{mM/l}$ (anhydrous)
- 7) *Ringer Locke* (*J. Physiol.* 18: 425, 1895 — *Zentralbl. f. Physiol.* 14: 670, 1900) quot. *Abderhalden* (Dittler).
 $K\ 2.68\ \text{mM/l}$ $Ca\ 1.81\ \text{mM/l}$
- 8) *Friedenthal* (*Arch. f. Anat. u. Physiol.* 550, 1903) quot. *Abderhalden* (Dittler).
 $K\ 4.02\ \text{mM/l}$ $Ca\ \text{---}$
- 9) *Tyrode* (*Arch. intern Pharmacodyn* 20: 205 1910) quot. *Abderhalden* (Dittler).
 $K\ 2.68\ \text{mM/l}$ $Ca\ 1.81\ \text{mM/l}$
- 10) *Göthlin* (*Skan. Arch. f. Physiol.* 12: 1 1902) quot. *Abderhalden* (Dittler).
 $K\ 1.34\ \text{mM/l}$ $Ca\ 0.585\ \text{mM/l}$
- 11) *Saika-Benda* (*Z. Biol.* 63: 11 and 531 1914) quot. *Abderhalden* (Dittler).
 $K\ 1.34\ \text{mM/l}$ $Ca\ 0.90\ \text{mM/l}$
- 12) *Heden und Feigl* (*Arch. int. de Physiol.* 3: 1, 1905—06) quot. *Abderhalden* (Dittler).
 $K\ 4.02\ \text{mM/l}$ $Ca\ 0.90\ \text{mM/l}$
- 13) *Adler* (*JAMA* 2: 9 og 752 1918) quot. *Abderhalden* (Dittler).
 $K\ 5.36\ \text{mM/l}$ $Ca\ 3.60\ \text{mM/l}$

Fig. 10. Table of K^+ / Ca^{++} Proportion in Different Artificial Tissue Fluids Used in the Literature.

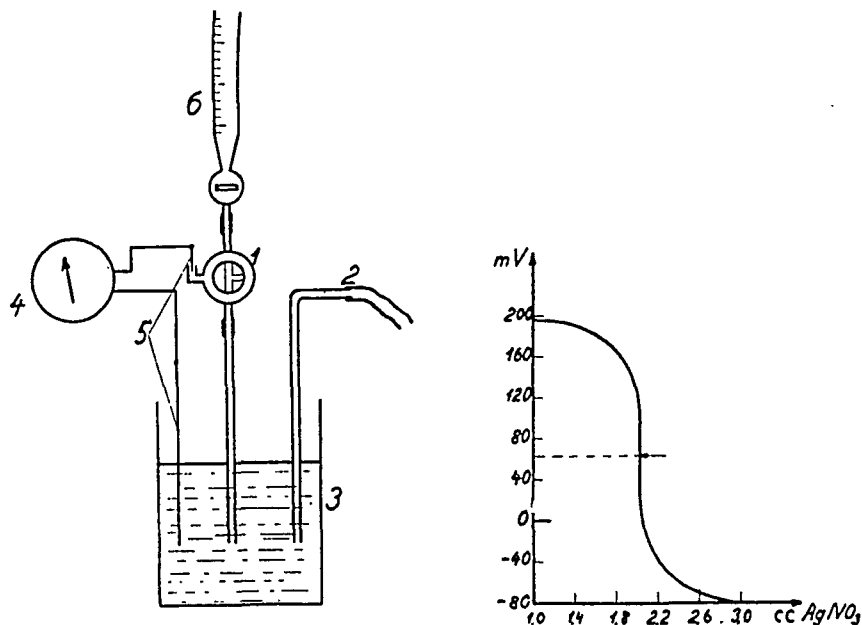


Fig. 11. *Lehmann's Method for Cl⁻-Estimation.*

- 1) Three-way stopcock with side tube for silver electrodes.
- 2) Glass tube through which a current of air is passed to mix the fluids in 3.
- 3) Vessel containing test sample dissolved in 5 ml 50 per cent CH₃COOH.
- 4) Valve potentiometer.
- 5) Silver electrode immersed in test solution.
- 6) Microburette containing 0.1 N AgNO₃ in HNO₃ solution.

The AgNO₃ is slowly added under constant aerating to the test sample in 3 and the EMF between the silver electrode in the AgNO₃ solution and the one in the test sample is measured at short intervals. The results of the EMF measurements will give a curve as shown above. The EMF will pass 0, when all Cl⁻ in the test solution is precipitated as AgCl and the amount of AgNO₃ added to make the potential = 0 corresponds to the amount of Cl⁻ in the test solution. The system is calibrated against a solution with a known Cl⁻ content, and it is now easy to calculate the content of Cl⁻ in any test sample with unknown content.

In the various experiments, solutions with different ion concentrations were used. Throughout the whole work these solutions were made in such a way, that it was possible, by mixing two standard solutions, to obtain the ion concentration required for any particular series of experiments. In the respective chapters a short summary will be given of the composition of the solutions used in the experiments described in that particular chapter. To make solutions with the same osmotic effect, but with a different ionic content, from the standard Ringer, it was necessary to interchange the electrolytes of which not only the molecular weights

were of importance, but also the relative ionic activity; the values given by *Heilbrunn* [1940] in the latest edition of his *General Physiology*, page 96, were used. In preparing bicarbonate solutions for the various working solutions with large calcium content, it was necessary to dissolve the bicarbonate in a small amount of solution before the addition of the calcium chloride, otherwise the calcium would be precipitated as calcium carbonate. All the other solutions could be prepared in the usual way, and buffered by the same bicarbonate solution, without causing any change of pH. To gain an insight into the action of standard Ringer's solution on the isolated nerve fibre, experiments were made to determine the survival time of a preparation at room temperature. The

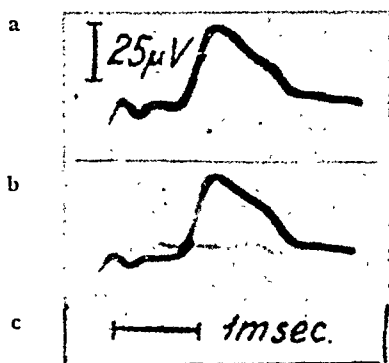


Fig. 12. Action Potential of Fibres Surviving 3 Hours.

a. Before. b. After 3 hours in Ringer Solution. c. Time in ms.

action potentials were completely unchanged over 2—3 hours, as shown in Fig. 12, even when the preparation was stimulated every minute with an automatic stimulating device; this seemed satisfactory in view of the fact that no preparation was used for more than 1½ hour and never stimulated as often as described. The experiment showed therefore that it was possible to work with a Ringer's solution containing no colloid; this was a great advantage as it was impossible during the war to obtain the special ion-free colloids [Polyviol] normally used for such purposes. Gum. arabic. was found not to be a suitable substitute for Polyviol, because it was necessary to dialyse every sample before use, as the gum. arab. itself contains large and inconstant amounts of ions.

The effect of temperature was studied by leading water of different temperature through the double glass floor in the chamber. The temperature of the fluid surrounding the nerve was measured with a thermocouple connected to a galvanometer [Fig. 3, 9], and with the second junction in a *Dewar's* flash filled with ice water, the same arrangement as that used by *Buchthal* [1936].

The anoxia experiments were made by passing hydrogen, purified from oxygen over red-hot sponge platinum, and saturated with water, into the two different parts of the chamber shown in Fig. 1 A and 1 B. It was important that the outlet tube was lower than the inlet tube, to ensure that the whole chamber was filled with hydrogen. As said before, details of technical procedures which are not mentioned in this chapter will be given in the various sections describing the experiments.

Description of method used for preparation of colloid Na-permutite ion exchanger.

25 g aluminium chloride is dissolved in 100 ml distilled water. To the solution is 10 normal sodium hydroxide slowly added, until the cloudy precipitate is again dissolved. 10 ml of this solution is diluted to 500 ml and 30 ml sodium silicate solution is added [25 g sodium silicate in 100 ml of water]. This will give a precipitate, and further precipitation will take place, when sodium chloride in crystalline form is added. The precipitate is separated from the solution and washed in distilled water until its reaction to litmus paper is neutral. The action of the exchanger can be checked by shaking a small amount of it in a few ml 1/100 normal calcium chloride solution. After shaking, the solution is passed through a calcium-free filter, and, if the exchanger is working correctly, no calcium oxalate will precipitate when a sodium oxalate solution is added.

CHAPTER 3.

THE SHAPE OF THE ACTION POTENTIAL UNDER PHYSIOLOGICAL CONDITIONS

WHEN small alterations in the amplitude and form of the action potential under various conditions have to be investigated, it is necessary to use single nerve fibre preparations to be sure that the recorded alterations are not due to differences in the number

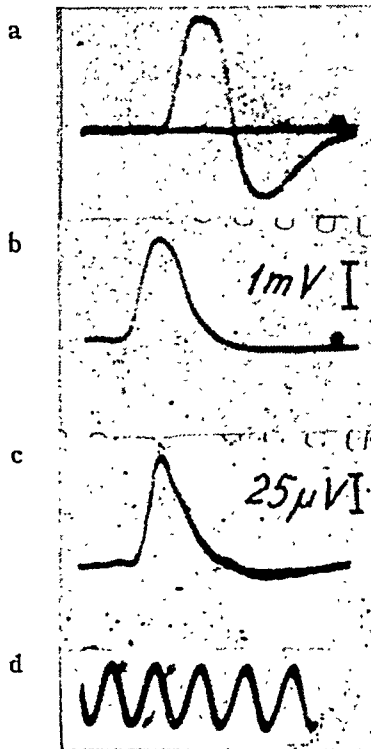


Fig. 13. Action Potentials from whole Nerve and from isolated Nerve Fibre.
a. Diphasic potential from whole nerve.
b. Monophasic potential from whole nerve. The spike has an ill-defined toppoint.
c. Monophasic potential from isolated fibre. The toppoint of the spike is well defined.
d. 1 ms. between time marks.

of fibres working, but are really due to changes in the activity of the single fibre. The technique described was therefore developed, and in this chapter a short account will be given of the action potential recorded in this way under "physiological" conditions.

Fig. 13 a, shows a diphasic action potential from a whole nerve.

Fig. 13 b, shows a monophasic action potential, recorded from a whole nerve with the end crushed, and the connective tissue removed. The last figure [13 c] shows the action potential obtained from the isolated fibre. It is noteworthy that here the action potential has a much better defined maximal amplitude, and a more easily recognisable positive after-potential than the potential from the whole nerve.

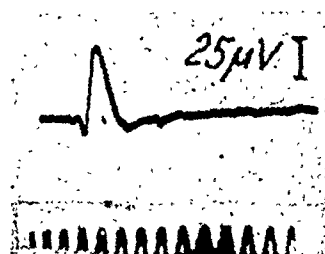


Fig. 14. Action Potential from a Nerve Fibre under "physiological" Conditions. (1 ms. between time marks) (Data see text).

Fig. 14 shows a representative single medullated fibre potential from the frog under physiological conditions [Ringer fluid as described pH 7.3, temperature 17°], and when analysed the results correspond well with the results obtained from a number of fibres under equal conditions. — The latency period + the conduction time [distance 13 mm] was 0.4 ms. [1 ms. = 1/1000 sec.] The ascending phase of the spike had a duration of 0.9 ms.

The duration of the descending phase of the spike + the negative after-potential was 1.1 ms., and the positive after-potential lasted for 4.2 ms. The maximal amplitude of the spike with an interelectrode resistance of 40.000 ohms was 75 μ V. [The frogs were caught in summer, and the records taken in September].

CHAPTER 4.

THE EFFECT OF ANISO-OSMOTIC SOLUTIONS ON THE NERVE FIBRE

THE experiments described in the following pages were made to gain some information about the permeability of the membrane surrounding the axis cylinder — or if one does not believe in the existence of a definite membrane — then to investigate the ability of the axoplasm to exchange ions with the surrounding fluid under aniso-osmotic conditions.

The plan of the experiments was to measure the diameter of the isolated nerve fibre under the microscope and observe the variations in its diameter when the fluid in which it was immersed was changed from ordinary iso-osmotic Ringer solution to various aniso-osmotic solutions. The diameter changes were taken as an indication of the ability of the fibre to take up or loose water and ions under experimental conditions. Simultaneously the action potentials were recorded in order to check to what extent, the function of the nerve fibre was affected by changes in intra- and extra-cellular water and ion content.

The solutions used consisted of ordinary Ringer modified for the purpose by alteration of its NaCl content. In order to ascertain that the results observed were purely osmotic, and not due to any specific effect of the sodium or chloride ions, five experiments were performed in which hypertonicity was obtained with glucose instead of sodium chloride. In some experiments the diameters of the fibres were measured in solutions rendered hypertonic by raising the potassium chloride concentration.

The specific potassium effect which was described by *Cowan* [1934] and others and discussed in the next chapter, abolished the action potential before any change in fibre diameter occurred,

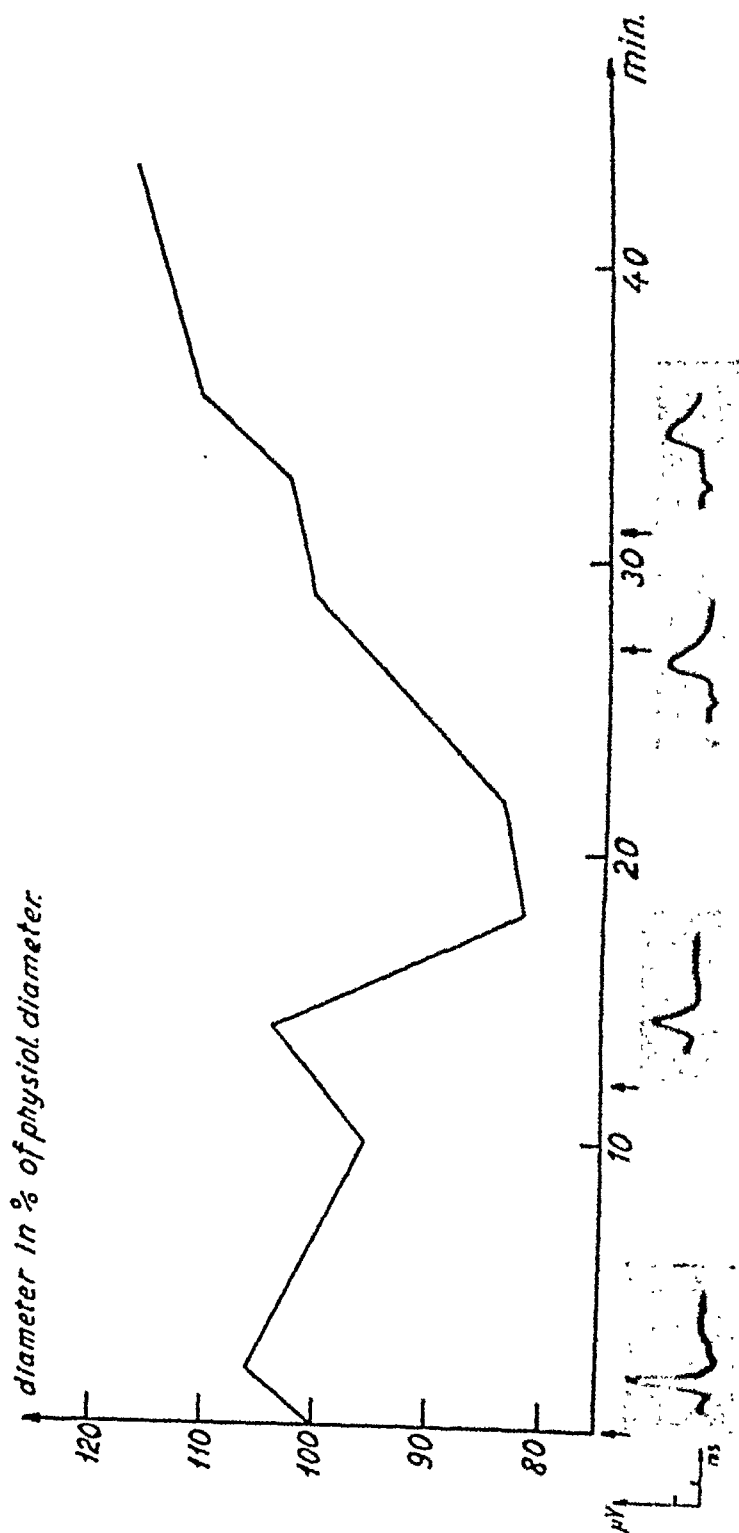


Fig. 15. Fibre Diameter of Nerve Fibre and its Action Potentials in Hyper-
tonic Solution.

Ordinate: Diameter in per cent of the physiological diameter.

Abscissa: Time in min. — Arrows indicate, when the potentials were recorded. (1.5 equivalent glucose.)

and hence the action potential could not be recorded simultaneously in these experiments, but the diameter curves did not differ from those in the experiments with raised potassium concentration in isotonic solutions, described Chapt. 5 below. This was in contrast to the results on muscles, described by *Boyle and Conway* [1941]. These authors found that when muscles were immersed in solutions with an increased amount of KCl, the volume of the muscles would only increase, if the solution was isotonic, but remain unchanged, if the solution was hypertonic.

In Fig. 15 the ordinate represents the percentage variation of the fibre diameter from that, measured in normal Ringer solution, while the abscissa is the time for which the fibre was immersed in hypertonic solution. [27.1 per cent glucose = circ. 1.5 molar]. The arrows indicate the time when the action potentials at the base of the curve were registered. [1.5 molar glucose solution is approximately equal to 0.84 molar NaCl solution in respect of osmotic activity, because NaCl is dissociated in two ions].

Fig. 16 shows the mean curves for hypertonic solutions obtained from all experiments, plotted in the same way.

Fig. 17 shows a nerve fibre in physiological "Ringer", and the

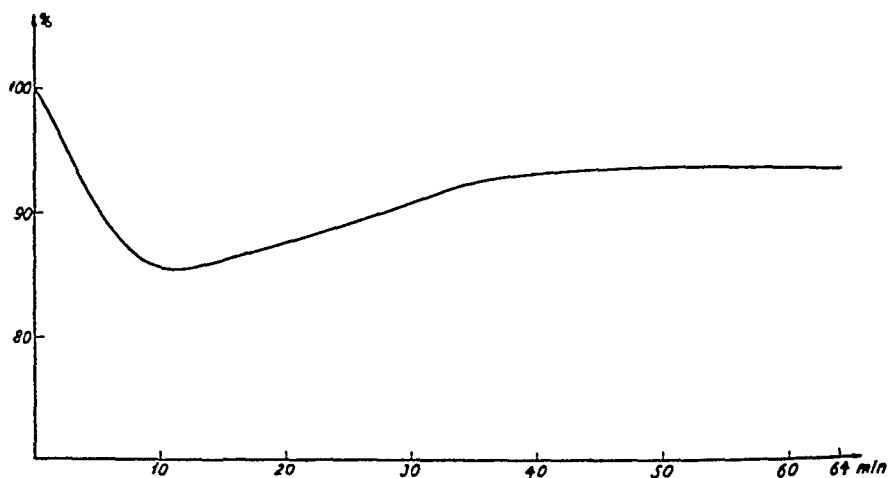


Fig. 16. Mean Curve of Diameter Variation of Nerve Fibres in Hypotonic Solution.

Ordinate: Diameter in per cent of the physiological diameter.

Abscissa: Time in min.

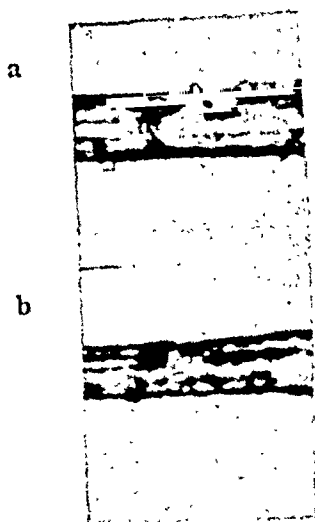


Fig. 17. Isolated Nerve Fibres in Isotonic and Hypertonic Solution. (Micro-photograph 650 \times magnification).

a. Fibre in isotonic solution. b. The same part of the same fibre after 15 min. in 1.5 equivalent glucose.

same fibre after 15 minutes in 5 per cent = 0.84 molar sodium chloride solution.

The following facts are apparent from the experiments:

[1] The nerve fibre diameter seems to be extremely insensitive to osmotic changes in the surrounding fluid. Only when the osmotic pressure falls to one-third of, or rises to seven times the physiological value can any changes be observed in the diameter of the fibres.

[2] The changes observed do not develop until the fibre has been immersed in the aniso-osmotic solution for 5 to 10 minutes. There is then a rapid change in diameter, of about + 15 per cent for hypotonic, and -15 per cent for hypertonic solutions, independent of their osmotic concentrations, if these are under 1/3rd or over 7 times the physiological value respectively [physiological value 0.1 molar NaCl].

[3] If the fibre remains in the aniso-osmotic solution, the diameter will return to its original value in about thirty minutes.

[4] When the fibre is immersed in hypertonic solution, its diameter will decrease, when it is immersed in hypotonic solution, the diameter will increase. The development of these diameter-changes according to time will, however, be the same in both instances.

[5] The fact that glucose may be exchanged for NaCl in these experiments with an identical result, shows that the intact nerve fibre membrane is impermeable to NaCl.

When the action potentials on the bottom of Fig. 15 are subjected to a more close analysis, it appears that the potential is only altered to a small degree; during the first 5—10 minutes, when also the diameter is unchanged, the amplitude has dropped, and the negative after potential is slightly prolonged. As soon as the changes in diameter are visible, the alterations in the action

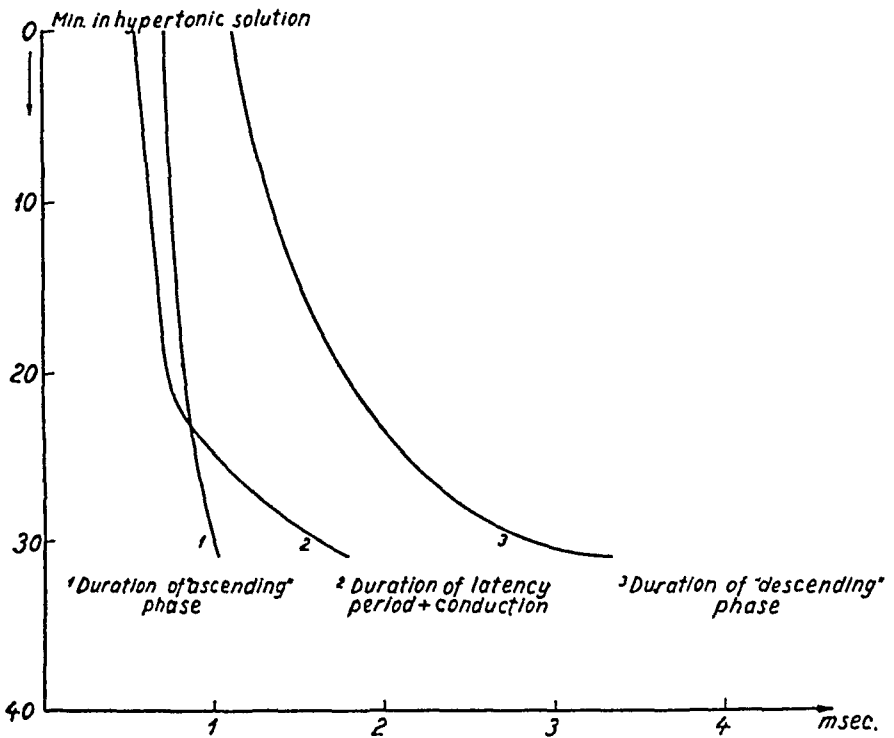


Fig. 18. Changes in Action Potential in Hypertonic Solution.

Ordinate: Time for which the fibre was immersed in the hypertonic solution.
 Abscissa: Duration in ms of the various parts of the action potential.
 Curve 1: Duration of the ascending part of the spike.
 Curve 2: Duration of the latency period + the conduction time.
 Curve 3: Duration of the descending part of the spike + the negative after-potential.

While the duration of the ascending fase of the spike is only influenced to a relatively slight degree, the conduction time and the after-potential are prolonged by 100 per cent after 30 min.

potentials also become more characteristic. These changes are irreversible.

Fig. 18 summarises the typical changes in the action potentials in an experiment with hypertonic solution. [27,1 per cent glucose = 1,5 molar]. The abscissa represents the duration in ms of various parts of the action potential, while the ordinate illustrates the time for which the fibre was immersed in the hypertonic solution. It appears that the duration of the latency period + the conduction time in the course of half an hour increases from about 0.5 ms to nearly 2 ms. [Curve 2]. [Unfortunately it was not possible with only one channel at disposal to differentiate between latency period and conduction time].

In the same 30 minutes the duration of the descending phase of the spike potential + the negative after potential increases from about 1.2 ms to about 3.4 ms, while the ascending phase of the spike only increases from 0.8 ms to about 1 ms. — The positive after-potential will disappear in the negative as the duration of the latter increases. It will be shown later that the effect produced on the action potential with hypertonic or hypotonic solutions are very much like the effect produced by low temperature, only that the later is reversible, while the former invariably leads to destruction of the conducting mechanism of the fibre.

After thirty to forty-five minutes the changes in the action potential mentioned, were fully developed, while at the same time the diameter had returned to its original value. For about two or three hours no further changes were observed, after which the fibre finally ceased conducting altogether, whether or not it was re-immersed in physiological Ringer solution. The changes in potential were of the same character whether the solution was hypertonic or hypotonic, and whether the hypertonicity was achieved by increasing the NaCl or the glucose content.

The relatively long time necessary to produce any change in the fibre diameter with strongly aniso-osmotic solutions indicates a low permeability, not only for glucose and NaCl, but even for water. When the change finally occurs, it is accompanied by an irreversible change in the action potential, indicating that the nerve has suffered permanent damage; this conclusion is further supported by the fact that, although the fibre may still be in aniso-

osmotic solution, its diameter returns to the original value and no further alteration of the osmotic properties of the surrounding fluid can now alter the diameter. The spontaneous return to the original diameter shows that the fibre is now freely permeable to water and to the ions concerned.

The water impermeability of the intact nerve membrane is in contradiction to the findings of *Boyle and Conway* [1941] for muscle membranes. It is also contrary to *Krogh's* findings on ganglionic cells [Personal comm. 1943], in which it was directly shown with heavy water that water freely passes the membrane. It disagrees with the findings of *Jacobs* [1934] for red blood corpuscle membranes in which it was shown that water freely passes in and out. The impermeability of the uninjured axis cylinder to water is in good accordance with the report by *Young* [1944] in which he states that a certain hydrostatic pressure inside the whole axis cylinder, maintained by the ganglion cell, is an important factor in the regeneration of cut fibres. It is perhaps not surprising that the nerve fibre has a much lower water permeability than a muscle, blood corpuscle and ganglion, the functioning of all of which is entirely dependent upon exchange of water soluble substances through their membranes. It must further be appreciated that the nerve fibre is only a highly specialized part of a cell, the body of which is permeable itself, as mentioned.

The detailed study of the changes occurring in the action potential when the permeability of the fibre is permanently altered is of interest, as it may give some information about the significance of the membrane permeability for the impulse propagation. The inclination of the ascending phase of the spike potential varies only to a proportionately small degree throughout the experiments, even when the change of diameter indicates that the permeability of the axis cylinder is irreversibly changed. This is not easy to explain, if one assumes [*Hill* 1932] that the potential is set up by the difference in permeability of the membrane to Na and K, permitting K to leak out without replacement by Na.

The principal changes in the action potential as the normal permeability of the nerve fibre is lost, are a decrease of conduction rate and an increase of negative after-potential. It is worthy of note that the conduction rate is decreased independently

of change in diameter, in contradistinction to the relationship found [Erlanger and Gasser [1937]] between nerve-fibre diameter and conduction rate under isotonic conditions.

In view of the experiments of *Graham* [1934] concerning the increased irritability of a nerve during the negative after-potential it was unfortunate that our electrical equipment did not permit exact measurement of irritability during the phase of increased negative after-potential. It is, however, hoped to investigate this point later. It would be important to find out, whether the damage from aniso-osmotic solutions to the surface of the axis cylinder which result in increased negative after-potential also results in increased irritability as this fact might illuminate the problem of some pathological rhythmic discharges.

Another question not included in this purely physiological investigation is the possibility of recording similar changes in the after-potential in other conditions in which the permeability of the axis cylinder is assumed to be altered — as for example, in different forms of neuritis. This might be of interest especially in B_1 -avitaminosis, where one is in need of a test showing, whether or not damage has occurred to the nerves of laboratory animals.

CHAPTER 5.

ISOTONIC SOLUTIONS CONTAINING INCREASED OR DECREASED AMOUNTS OF KCl

MAC DONALD and later *Cowan* [1934] and *Helen Treadway-Graham* [1933] described the potassium effect on the action potential as the production of a reversible blocking, gradually increased with increasing K^+ -concentration.

The experiments reported in this chapter were made to determine whether the shape of the action potential as well as its amplitude were affected by potassium. Previous investigations were made on whole nerves while our experiments were carried out on isolated fibres to ensure that progressive blocking really took place in the single fibre and was not an effect caused by a decreasing number of active fibres in the nerve trunk.

Two modifications of the Ringer solutions were made with the following content:

<i>Modification.</i>	<i>I</i>	<i>II</i>
NaCl	6.857 g	0
KCl	0	8.777 g
CaCl ₂ [anhydr.]	0.110 g	0.110 g
Glucose	0.200 g	0.200 g
Aq. dest.	1000 g	1000 g

Isotonicity was maintained in both stock solutions in spite of their different KCl content, by altering their NaCl content appropriately. By mixing various proportions of the two fluids it was possible to obtain any desired KCl concentration from 0 to about 43 times the physiological KCl content maintaining iso-osmotic conditions.

By buffering with NaHCO_3 as described in chapter 2 for normal Ringer and by aerating with O_2 containing 1 percent CO_2 , the pH was maintained at a constant value.

The results of the experiments on the fibre action potential with variation of the K^+ -concentration in the surrounding fluid under iso-osmotic conditions can be summarised as follows:

1) Treating of the fibre with a solution prepared without KCl does not in any way alter the action potential [Probably enough KCl *always* is adhering to the fibre surface to maintain a minimal concentration].

2) When the KCl concentration is raised in the surrounding fluid, the action potential will decrease. Only a relatively small decrease is observed, when the concentration is increased 5 times. Between 5 and 8 times a critical value — different for individual fibres — will be found where the potential is suddenly reduced

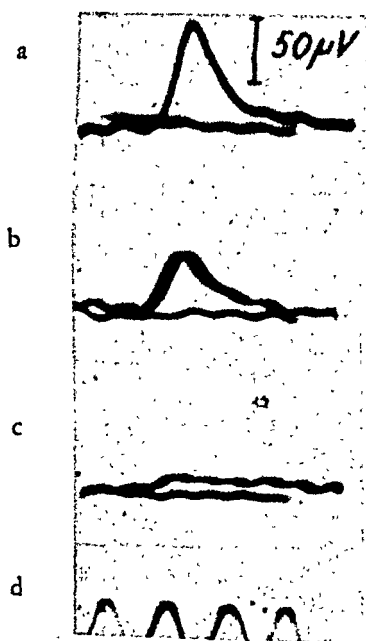


Fig. 19. Action Potential from Nerve Fibre in Isotonic Potassium Chloride Solution.

- a) Potential in Ringer Solution (2,66 mM K^+).
- b) Potential in an isotonic solution with a K^+ concentration of 5 times the physiological.
- c) The potential in 6 times the physiological K^+ concentration.
- d) 1 ms. between time marks.

to 0, and the nerve blocked. The KCl concentration in our "physiological" Ringer was 2.66 millimol.

3) The shape of the reduced spike-potential as well as of the after-potentials seem to be unaltered. We were not able to confirm *Graham's* [1933] findings on whole nerves of a prolonged negative after-potential.

4) *All the changes described develop instantaneously, i. e. within $\frac{1}{2}$ —1 sec., and they are within limits [K^+ -concentration below 10 times the physiological one, and immersion below 15—20 minutes], completely reversible.*

Fig. 19 shows potentials in normal Ringer, in fluid with 5 times

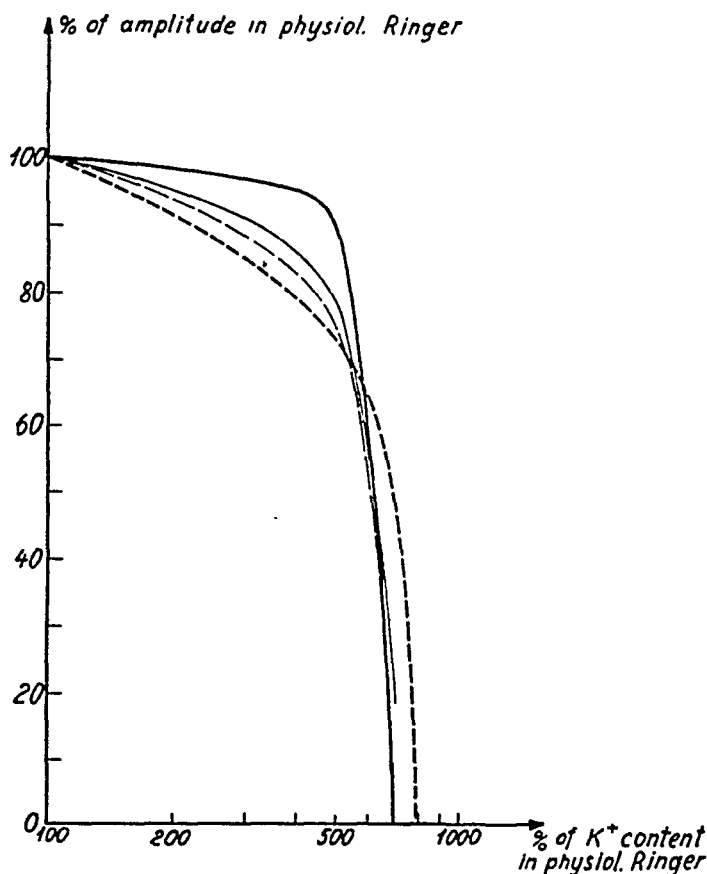


Fig. 20. Amplitude of Action Potential as a function of Concentration. Ordinate: Maximal amplitude of spike in per cent of the amplitude in Ringer Solution with a physiological K^+ concentration. Abscissa: K^+ concentration in per cent of the concentration in physiological Ringer solution 0.2 per 1000 ml (logarithmic scale).

the normal K^+ -concentration and in fluid with 6 times the normal K^+ -concentration.

In Fig. 20 the percentage amplitude is plotted along the ordinate, while the abscissa represents the various concentrations employed in per cent of the normal concentration, and plotted on a logarithmic scale. It can be seen from the figure that the curves are not exponential ones as should be expected from a pure concentration effect, but that there is, as described above, a critical value of the K^+ -concentration in each experiment, where the potential is reduced to 0. This is interesting compared with the facts, described below that we found an increase in diameter of fibres, immersed in isotonic solutions with a K -concentration of about 10 times the normal or higher, while *Hodgkin* (1946) found a decrease of fibre resistance in fibres immersed in isotonic solutions with K^+ -concentration of the same magnitude.

It should be stressed once more that the resistance between the leading-off electrodes was kept constant, when measuring the relative amplitudes. When the diameter of the fibre was measured simultaneously, no change could be observed in fibres exposed to potassium concentrations which did not render the blocking irreversible [approximately 10 times the physiological concentration].

In view of the fact that *Boyle and Conway* [1941] find the muscle membrane freely permeable to potassium and also the fact that *Cowan* has found that the nerve is freely permeable to K^+ , experiments were made in which the fibre-diameter was registered with the fibre immersed in an *isotonic* solution with 10—15 times the physiological potassium concentration. Fig. 21 is a mean curve of the percentage diameters measured.

In contrast to the osmotic experiments mentioned above, it can be seen that the diameter increases about 12 per cent in the isotonic solution, suggesting that the potassium chloride has migrated into the fibre substance. After about 30 minutes the diameter returns to its original value. The process is a slow one, and it must be appreciated that *the action potential is irreversibly extinguished in the weakest of these solutions before a change in diameter can be observed*. It was, therefore, concluded that under the present experimental conditions when the fibre was able

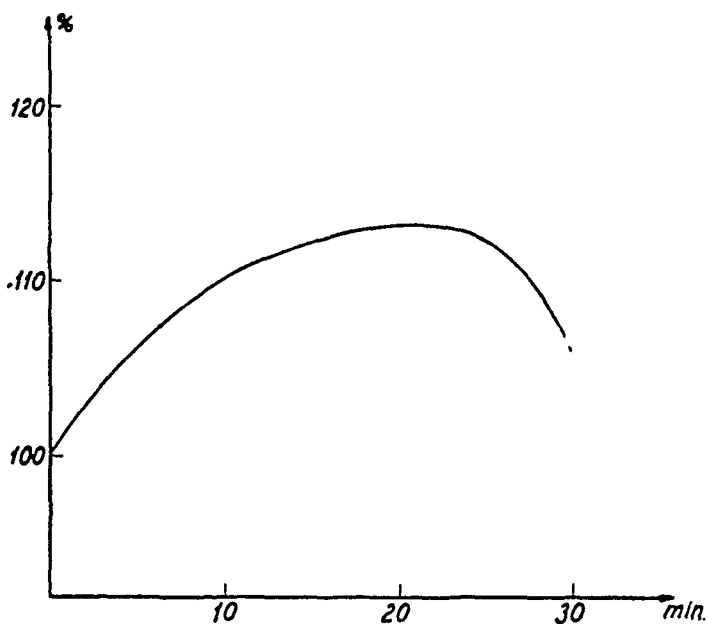


Fig. 21. Diameter of Nerve Fibre in Isotonic Potassium Chloride Solution.

The figure represents a mean curve of the diameter variations in a series of experiments where the diameter of the fibre was measured after immersion in isotonic solutions containing from 10—15 times the physiological amount of K^+ . In all these experiments the action potentials were irreversibly extinguished after 4—5 min. Ordinate: Diameter in per cent of physiological diameter. Abscissa: Time for which the fibre was immersed in the K^+ solution in min.

to take up potassium chloride from the surrounding fluid, it was no longer able to conduct impulses. This is in agreement with the results from a control of *Boyle* and *Conway's* experiments, made by *Buchthal* and *Folkow*, in which muscle fibres proved to be irreversibly unexcitable under the conditions described by the former authors.

In relation to this slow potassium-uptake of a nerve-fibre immersed in an isotonic solution with a raised potassium-content it might be mentioned that *Hodgkin* and *Rushton* recently [personal communication 1945], measuring electric constants of a crab nerve fibre by the deformation of a square wave, found a slowly decreasing fibre membrane resistance in solutions with a high potassium content.

In two experiments $RbCl$ was used instead of KCl , and identical results were obtained.

Like *Cowan* [1934] we investigated the antagonistic effect on

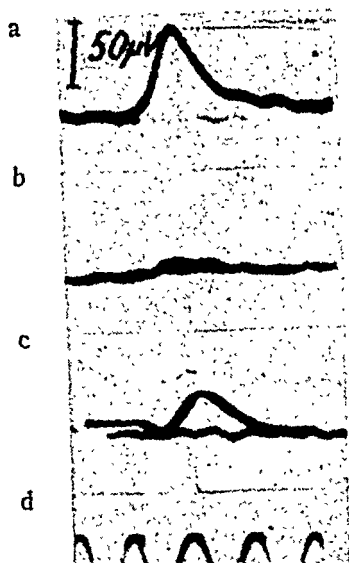


Fig. 22. Ca^{++} Effect and K^+ Effect on the Action Potential of Isolated Nerve Fibres.

- a) Action Potential in physiological Ringer solution.
- b) Reduced action potential in a solution with a K^+ concentration of 6 times the physiological value.
- c) Blocking partly abolished in a solution containing $6 \times$ the physiological amount of K^+ and 6 times the physiological amount of Ca^{++} .
- d) Distance between time marks 1 ms.

the fibre action potential af Ca^{++} to K^+ . Fig. 22 is a typical example. It may be seen from the Fig. that a rise of CaCl_2 in the same proportion as the KCl rise caused the action potential to reappear, with about one-third of its original amplitude and its original shape, at the point where it was blocked by the KCl rise. The Ca^{++} action is instantaneous and reversible as is the K^+ action.

By application of a potassium-free solution it was of course, as described above, impossible to remove all potassium from the fibre surface, and we therefore tried to remove the rest by the method of absorption. In a paper by *Szent-Györgyi* [1942] it was quoted that experiments had been performed by *Geréndas* and *Varsanyi* in which K^+ had been inactivated in myosin threads by Na:Al silicate [permutite], causing contraction of the threads. Accordingly the effect of an ion exchanger on the action potential and the diameter of a nerve fibre was investigated. Three different permutites were employed in six experiments. The first two of these were an

organic [Beolite] and an inorganic [Natrolite] preparation, both of which were produced commercially by the Swedish firm "Permutite". For our purpose they were treated with a saturated NaCl solution for 24 hours, then rinsed frequently in distilled water for 24 hours, until tests with silver nitrate showed that the water was completely free of Cl^- . Then they were crushed and mixed with Ringer solution until a fine clay was obtained, which was packed round the nerve fibre. As these experiments were completely negative, no change in either action potential or diameter being observed after several hours exposure, a colloid Na-permutite was prepared in the laboratory in the way described on page 33; this also was without any effect, even when it was soaked in isotonic glucose solution instead of Ringer; this was done in two experiments to prove that the lack of effect was not due to "blocking" of the permutite by ions from the Ringer solution.

The negative results of the permutite experiments may be due to the fact that the ion exchanger on account of the impermeability of the fibre does not come in contact with the ions concerned.

The experiments described above with iso-osmotic solutions containing increased K and Ca differ from the experiments with hyper- and hypotonic solutions described in the previous chapter. The differences can be summarised as follows: —

[1] The fibre diameter remains constant as long as the potassium effect is reversible.

[2] The amplitude of the action potential is altered; the effect is uniformly on spike and after-potentials.

[3] The alterations are fully developed within half a minute.

CHAPTER 6.

EFFECT OF OTHER CHEMICAL CHANGES UPON THE ACTION POTENTIAL

I. Changes in Ca^{++} Content.

Lehmann [1937] found that exclusion of CaCl_2 from the fluid surrounding a mammalian nerve caused a lowered threshold with spontaneous discharges, decreased negative after-potential and increased positive after-potential. The same effect could be produced by a high pH or by de-ionisation of the calcium with citrate. Exclusion of potassium from the solution caused a reversal of these effects. None of these changes appeared until the nerve had been exposed to the solution for about an hour. *Lehmann's* experiments were carried out on whole nerves, and it was therefore difficult to ascertain how much the Ca^{++} effect was delayed by diffusion through connective tissues. *Lehmann* has not discussed the effect of lack of Ca^{++} on the spike potential. *Graham* [1933] found the same changes in whole nerves of frogs, as *Lehmann* had found in the mammalian nerves, but also there it is impossible to judge the activity of the single fibre from experiments on whole nerves.

We examined the effect of prolonged exposure of single nerve fibres from frogs to fluids with a non-physiological content of ionised calcium. If the calcium content of the fluids was decreased, or if they contained approximately 1 per cent Na citrate or Na oxalate in Ringer, the action potential remained normal for 40 to 60 minutes, then changes similar to those observed by *Lehmann* slowly developed. The amplitude of the positive after-potential increased, and rhythmic discharges developed; the spike potential remained of unaltered shape, but its amplitude increased [Fig. 23].

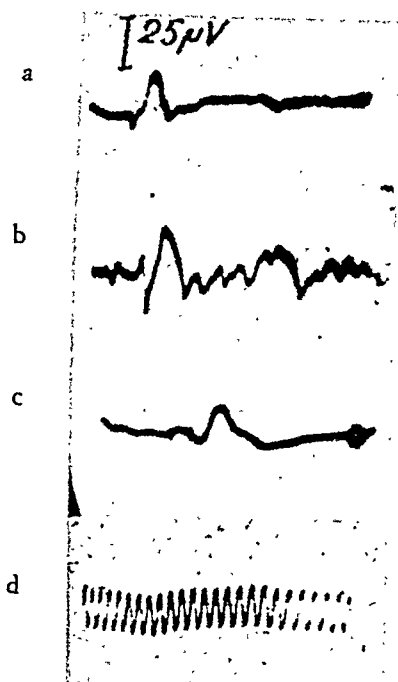


Fig. 23. Effect of Sodium Citrate on Nerve Fibre and Ca^{++} Effect

- a) Potential in physiological Ringer.
- b) Potential after 40 min. immersion in 1 per cent isotonic sodium citrate solution.
- c) After 30 min. in a solution containing 10 times the physiological amount of Ca^{++} .
- d) Distance between time-marks 0.5 ms.

These changes continued if the nerve was washed with Ca^{++} -free Ringer, but disappeared 15 minutes after the nerve was re-immersed in fluid containing Ca^{++} in physiological or higher concentration. If the nerve remains for 20 to 30 minutes in a solution containing ten times the physiological CaCl_2 , which was in our experiments 1 millimol, the maximum amplitude of the action potential fell, but the negative after-potential increased its amplitude so that the whole potential curve had a flattened appearance, with two negative peaks, in contrast to the irreversibly prolonged negative after-potential in the experiments with aniso-osmotic solutions [Fig. 23c].

It is interesting that all changes described in this chapter take from twenty to thirty minutes to manifest themselves contrary to the instantaneous potassium effect described in chapter 4, and

its immediate response to increase in calcium ions. This suggests that while the latter is an expression of interaction between the ions outside the axon and those in the surface layer, the phenomena described in this chapter imply a change of ion concentration inside the fibre.

Fig. 23, as mentioned, shows the rhythmic discharges, produced by citrate. During the long exposure of the nerve it was not possible to prevent neighbouring fibres from losing Ca^{++} and hence discharging spontaneously. The records of spontaneous discharges are, therefore, not necessarily those of a single fibre.

II. Magnesium.

Narcosis with magnesium sulphate has been known for over a century, but the exact mode of its action on different parts of the nervous system has only recently been investigated. *Hahn* [1910] and *Wiechmann* [1920] were not able to decide at what level it had its effect, while *Essen* [1931] and *Cloetta, Fischer and Loeff* [1942] stated that the Mg^{++} -ion acted centrally and on the motor endplate, the nerve itself not being affected. These

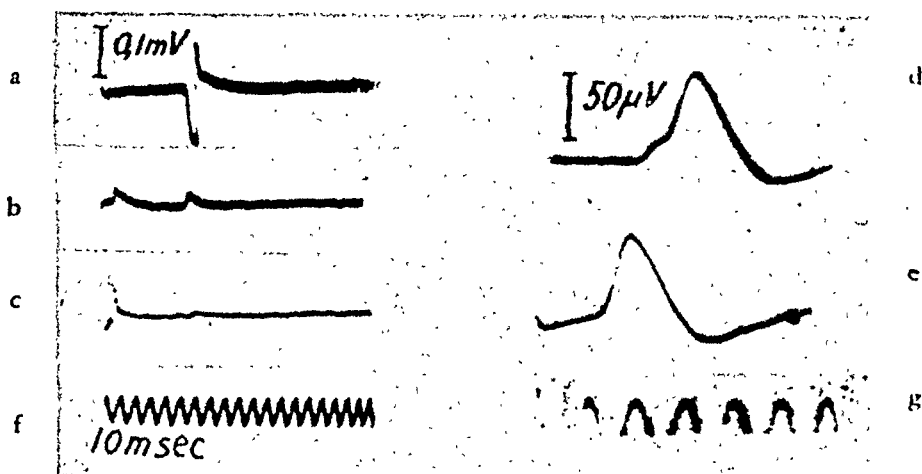


Fig. 24. Effect of MgSO_4 on Action Potential.

- a) Muscle action potential (stimulated through its nerve).
- b) Muscle action potential after 20 min. application of 800 mg per cent MgSO_4 solution.
- c) After 60 min. stay in 800 mg per cent MgSO_4 (time in ms.).
- d) Nerve fibre potential in physiological Ringer.
- e) Nerve fibre potential after 3 hours in 800 mg per cent MgSO_4 solution
- f) Distance between time-marks 10 ms.
- g) Distance between time-marks 1 ms.

authors made, however, no records of action potentials. *Greville* and *Lehmann* [1943] suggested that there was also an action on the muscle fibre of Mg^{++} . Parallel with work on motor end plates from this laboratory by *Lise Engbæk* we therefore investigated magnesium-sulphate action on our single nerve-fibre preparations. This was done in four experiments, of which Fig. 24 is an example. Solutions containing 8.3 — 16.6 — 41.5 — and 66.4 millimol $MgSO_4$ [100—200—500 and 800 mg per cent] in Ringer were tried without effect. After two hours in 66.4 millimol [800 mg per cent] iso-osmotic magnesium-sulphate Ringer the action potential of a nerve fibre was completely unchanged while after one hour the potential in the muscle fibre [stimulated through the nerve] had disappeared. The muscle fibre was still directly excitable.

III. Alterations in pH.

In a series of experiments the effect on the action potential of alterations of the pH in the surrounding fluid was investigated. It was surprising that even considerable changes in pH [pH 5 or pH 9] were without any effect over a period of 15 to 20 minutes, however after 45 to 60 minutes complete agreement with *Lehmann's* results on whole nerves was obtained. *Lehmann* found that high pH had the same effect on nerve-bundle actions potentials as loss of Ca^{++} , producing rhythmic oscillations.

In two experiments the effect of alteration of pH upon the potassium effect described in Chapter 4 was investigated, but the instantaneous reversible blocking produced by raised KCl concentration is unaltered independent of the pH of the KCl solution. This is interesting as it has been suggested that K^+ penetrates a hypothetical membrane together with dissociated phosphate or in combination with an ampholytic ion during propagation of excitation. It might, therefore, have been anticipated that this diffusion of K^+ would have been influenced when pH changes altered the dissociation of the relevant anion. The diameters of the fibres were not measured in any of these experiments.

IV. Acetylcholine.

The effect of acetylcholine as a transmitter of the nerve impulse to the muscle fibre was well established in work on whole muscle

[Dale, Feldberg and Vogt [1939]], Brown [1936], and isolated, motor end-plate muscle-fibre preparations [Buchthal and Lindhard [1939]]. For the central nervous system acetylcholine seems to have a stimulating action [Bouquet and Bremer [1937], Moruzzi [1939], Chang and Coworkers [1938]]. It has been suggested by Fulton and Nachmansohn [1943] that acetylcholine might be important also for impulse conduction in the peripheral nerve. "This new conception is based on two lines of evidence". [Fulton [1943]] : [1] The relation between electrical potentials and acetylcholine formation in the electric organs of *Torpedo marmorata*, *Gymnotorpedo occidentalis* and *Electrophorus electricus* [Nachmansohn, Cox, Coates and Machado [1942]]. [2] The presence of acetylcholine in the nerve fibre membrane.

Boel and Nachmansohn [1940], Lorente de Nó [1938] and Lissak [1939] also claim that acetylcholine is liberated from nerves during activity. With regard to point 1, it can be stressed that the electric organs developmentally as Fulton himself has pointed out (see also Lindhard 1931) are homologous to muscle motor end-plates and the co-ordination described between acetylcholine formation and action potential is therefore not surprising, nor does it prove anything about the behaviour of the peripheral nerve.

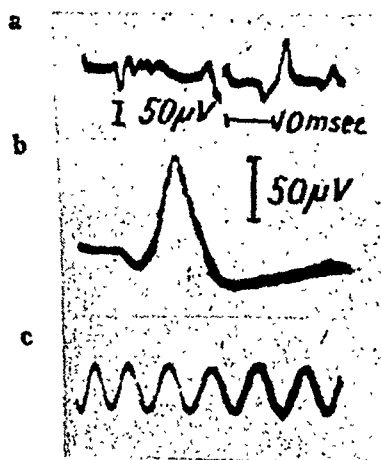


Fig. 25. Acetylcholine Effect on Action Potentials of Muscle and Nerve.

- a) Spontaneous discharges in muscle fibre bundle after one drop acetylcholine solution (concentration $1 \mu\text{g}$ per ml).
- b) Normal action potential without spontaneous discharges from nerve fibre immersed for 30 min. in the same solution.
- c) Distance between time marks 1 ms.

Concerning [2] amongst others, investigations by *MacIntosh* [1941] have shown remarkable difference between acetylcholine concentrations in the grey and white substances of the brain as well as between the synapse and the nerve fibre [*Nachmansohn* [1939]].

However, we found it necessary to re-investigate the effect of acetylcholine on our single fibre preparation. Fig. 25 shows the negative result of an experiment in this group. The fibre in this case was exposed for 25 min. to Ringer solutions, containing 1 μ g, 0.1 μ g and 0.01 μ g acetylcholine per ml, stabilised with 5 per cent Na_2HPO_4 . As a check on the activity of the acetylcholine solution it was tried in the concentration 1 μ g per ml on a frog muscle and had the effect shown in Fig. 25 b.

The reason why acetylcholine has no influence on the nerve fibre might of course be that it cannot penetrate the fibre membrane, but even when applied to a cross section of the fibre no excitation was recorded, although the fibre was still excitable electrically.

It is difficult to explain the discrepancy between the report by *Lissak* [1939] that acetylcholine is liberated from nerve fibres during activity and our inability to stimulate fibres with acetylcholine.

Taking all experimental facts into account it seems most likely to us that the stimulating action of acetylcholine is limited to the nerve cell and the motor end plate, and that the potential generated in the nerve cell is conducted along the nerve fibre by a different mechanism.

CHAPTER 7.

THE ACTION POTENTIAL OF A NERVE FIBRE DURING ANOXIA

THE very low oxygen consumption of nerve fibres led to the dogma at the end of the last century, that the nerve did not use oxygen [*Bernstein* [1877], *Wedensky* [1884], *Bowditch* [1890], and *Lambert* [1894]]. In the beginning of this century it was, however, shown that the nerve used oxygen, [*Thunberg* [1909]], and could be fatigued [*Garten* [1903], *Brodie and Halliburton* [1902]]. This view was further established by the investigations of *Haberlandt* [1910, 11]. He stated that the conduction rate was lower in a CO₂-atmosphere, and he also pointed out that the nerve was more easily fatigued in the neighbourhood of the excitation electrodes. Further investigations have been made in *Hill's* laboratory by *Levin* [1927], *Furusawa* [1929], *Gerard* [1927] and *Meyerhof* [1929]. Lately an apparatus for studying the asphyxiation of nerves has been described by *Shapiro* [1938]. However, from these various papers it is not clear in what proportion the excitability or the impulse conduction are affected by lack of oxygen. This problem was therefore investigated [*Buchthal and Hertz* [1945]].

Oxygen was removed from the two sections of the chamber in the way described in Chapter 2. With asphyxiation of the stimulated region of the nerve an average of 34 minutes was necessary to suppress activity, while it took 70 minutes on average when asphyxiation was limited to the part of the chamber containing the leading-off electrodes. Fig. 26 represents the action potentials obtained in a typical experiment. The action potential reappears immediately after readmission of oxygen but without a positive

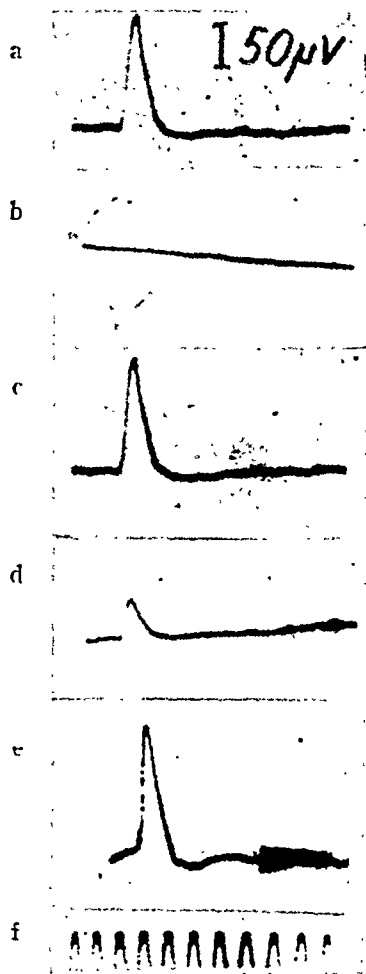


Fig. 26. Effect of Lack of Oxygen on the Action Potential.

- a) The action potential of a nerve fibre in atmospheric air.
- b) The action potential extinguished after the fibre has been placed in hydrogen for 70 min.
- c) The action potential reappears as the stimulated part of the fibre is again exposed to atmospheric air (part of chamber A Fig 1 open). The remaining part of the fibre is still in hydrogen.
- d) The action potential is again being extinguished after further 50 min. with the conducting part in hydrogen. The potential amplitude has decreased, and the positive after-potential has disappeared.
- e) After 15 min. in atmospheric air the action potential has regained its original size and shape.
- f) Distance between time marks 1 ms.

after-potential, as described by *Lehmann* 1937. After 15 minutes in oxygen it has regained its original shape.

During anoxia; aneurine in a neutralised solution or adenosine triphosphate in a solution strong enough to provoke muscle activity

[*Buchthal et al.* [1944]] were applied to the fibre but were without effect on the extinguished action potentials. This is interesting in view of the fact that aneurine [diphosphothiamine] was considered by *Mann and Quastel* [1940] to be important in acetylcholine synthesis in nerve while adenosine triphosphate was thought by *Nachmansohn, Cox, Coates and Machado* [1943] to be the source of energy for the action potential of the electric organ of *Electrophorus electricus*. These two agents were likewise without effect on the action potential of a single fibre in the presence of oxygen.

CHAPTER 8.

FIBRE ACTION POTENTIAL AND TEMPERATURE

ALL the experiments hitherto described in this paper were performed at 19°C .

In the present chapter the dependence of the fibre action potential on temperature changes will be examined. The effect of temperature on nervous activity has been studied by *Bernstein* [1902], and *Bernstein* and *Tschermak* [1906]. The temperature effect on action potentials of whole nerves has been investigated by several authors [*Amberson* [1930] — *Gasser* [1931] — *Erlanger and Gasser* [1937]]. *Gasser* has described the temperature coefficient of the amplitude of a nerve spike potential and found rapid variations of amplitude with temperature changes as in a purely physical system.

Our experiments were carried out on our usual single fibre preparations. Frogs caught in summer were used, and the investigations

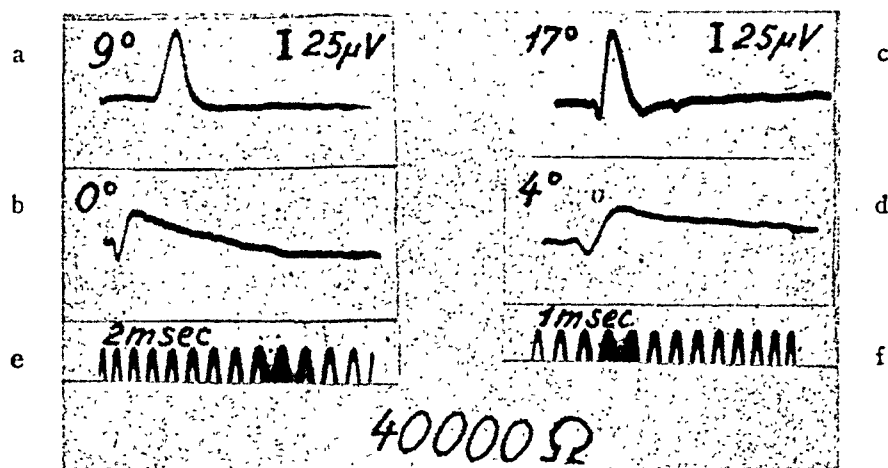


Fig. 27. Temperature Effect on Action Potential.

- The action potential of a nerve fibre at 9°C .
- The action potential of the same fibre at 0°C . Time 2 ms.
- The action potential of a nerve fibre at 17°C .
- The action potential of the same fibre at 4°C . Time in ms.
- Distance between time marks 2 ms.
- Distance between time marks 1 ms.

were made in September. From a bottle of hot water and a bottle of ice water, water of any intermediate temperature could be obtained by mixing. The water was led through the double floor of the chamber while the temperature in the Ringer fluid round the nerve was recorded by means of a thermocouple, as described in chapter 2. When the temperature was stabilised, action potentials were recorded. Fig. 27 shows typical potentials at various temperatures. The Fig. illustrates that the following changes take place when the temperature drops: [1] The conduction rate goes down [2] The spike amplitude decreases [3] The spike develops its maximum amplitude more slowly, the greatest changes taking place in the after-potential, which is prolonged. Just before the fibre stops conducting, the negative after-potential does not return to the isoelectric line after a spike. The changes in the action potential at low temperatures are almost similar to the effects of aniso-osmotic solutions, described in chapter 3. The temperature changes are, however, reversible between 0° and 30° , and develop instantaneously. Fig. 28 shows the temperature dependence of the amplitude in per cent of the maximal amplitude [cf. *Erlanger and Gasser, 1937, Fig. 72*].

Fig. 29 gives the relation between the temperature [in centigrades along the ordinate] and the duration of 1) latency period + conduction time, 2) ascending phase of action potential, and 3) descending phase + negative after-potential, all plotted in ms along

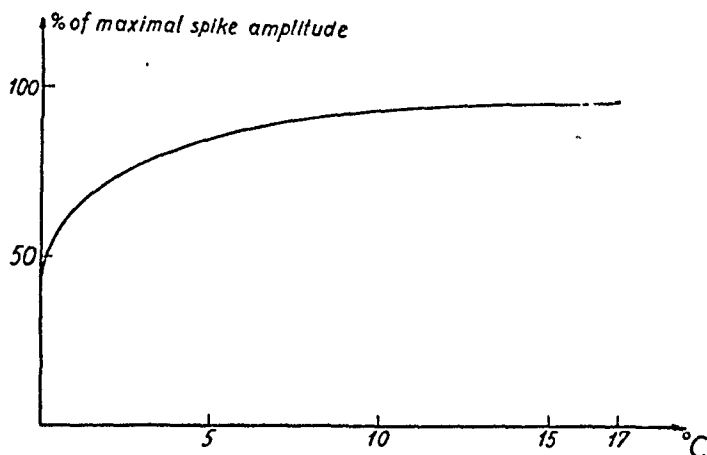


Fig. 28. Temperature Effect on Amplitude of Action Potential. Ordinate: Amplitude in per cent of maximal amplitude. Abscissa: Temperature in degrees C.

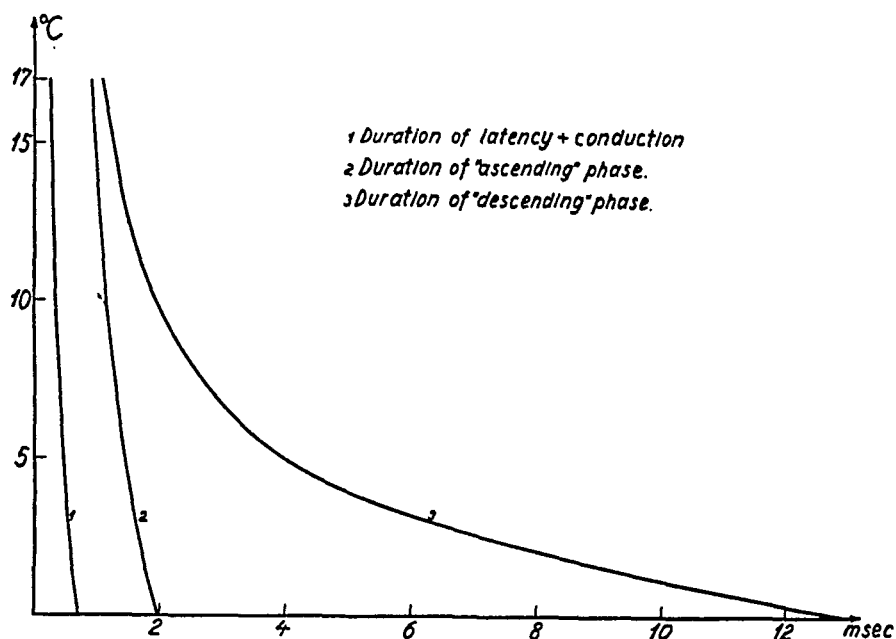


Fig. 29. Temperature Effect on Duration of Various Parts of the Action Potential.

Ordinate: Temperature in degrees C.

Abscissa: Duration in ms.

Curve 1: Duration of latency period + conduction time

Curve 2: Duration of ascending part of spike.

Curve 3: Duration of descending part of spike + negative after-potential.

The negative after-potential is influenced by temperature to a much higher degree than any other part of the action potential (Conf. Fig. 18).

the abscissa. It can be seen from the curves that the alterations in the duration of the various parts of the action potential at low temperatures are almost similar to those produced by immersion of the fibre in aniso-osmotic solutions, [Fig. 17], only that the effect of temperature is reversible, while the osmotic effect is irreversible. A possible explanation of the similarity will be given in the conclusion.

Bélehrádek [1935] has made calculations on various temperature-coefficients of biological processes. *Buchthal and Lindhard* [1936] described the temperature coefficient of the muscle and motor end-plate potentials, and as mentioned above, *Gasser* [1931] described the temperature coefficient of the spike amplitude of a whole nerve.

Realising the limited value of an estimation of a temperature coefficient for a process which has not a strictly linear temperature

dependence, we calculated the coefficient Q_{10} for a temperature experiment on a single nerve fibre. Q_{10} is described as:

$$Q_{10} = \left(\frac{P_1}{P_2} \right)^{\frac{10}{t_1 - t_2}}; \text{ the following formula was used:}$$

$$\log Q_{10} = \frac{10 (\log P_1 - \log P_2)}{t_1 - t_2};$$

t_1 and t_2 were the highest and lowest temperature used in the experiment. P_1 and P_2 were the corresponding values of a) the percentage amplitude in percent of the amplitude at the highest temperature t_1 [17°C], or b) the reciprocal value of the duration in ms of the various phases of the action potentials. The resulting Q_{10} 's are given below.

1) Spike amplitude in percent of the amplitude at 17°C $Q_{10} = 1.69$

2) "Conduction rate" = $\frac{1}{\text{Duration in ms of latency period} + \text{conduction}}$
 $Q_{10} = 2.03$

It must be pointed out that the value given for Q_{10} here is Q_{10} for the conduction rate as the latency period is not affected by the temperature changes because the excited part of the nerve was kept under constant temperature [see Fig. 1].

3) $\frac{1}{\text{Duration in ms of ascending phase of spike}} Q_{10} = 1.19$

Curve 3 Fig. 29 shows the temperature dependence of the negative after-potential. If the temperature is raised from 0°C . to 17°C ., the duration of the negative after-potential will fall from 13 ms to 1.1 ms; Q_{10} has not been calculated, on account of the form of the curve. The values for the positive after-potential are not introduced in Fig. 29 because the estimations were not accurate on our single fibre preparations, giving a low voltage, but in an experiment where the temperature was raised from 9°C . to 17°C . the duration of the positive after-potential fell from 8.8 ms to 4.2 ms, giving a Q_{10} of ca. 2.6.

It appears that the negative after-potential has a higher temperature dependence than any other part of the action potential, which corresponds with the theory presented in chapter 9 that the reorganisation of the membrane system, after the impulse has passed, is a more complicated process than the setting up of the impulse.

CHAPTER 9.

CONCLUSION AND PLAN FOR FURTHER WORK

WHEN part of the results described in this paper were first presented to the Physiological Society in London [Feb. 1945] the author had a very useful discussion with *S. L. Cowan*, about the permeability of the nerve membrane to potassium ions. The author thought to have shown that the permeability of the living unexcited axis cylinder to potassium was very low, while *Cowan* on the experiments mentioned above, based the conviction that potassium ions were able to pass the nerve membrane freely under all conditions, but, owing to some chemical mechanism, were only let out during excitation. In chapter 4 of this paper it was shown that the intact axis cylinder is impermeable to water, a view which is supported by the investigations of *O. H. Schmitt* [1938], and some experiments by *Hodgkin and Rushton* [personal communication 1945]. It was concluded from these investigations that the nerve fibre, during the development of the local potential, is charged as a condenser, which proves that the nerve membrane has a relative high dielectricity constant. Experiments with the same purpose were already carried out 1935 on single muscle fibres by *Buchthal*, but while *Hodgkin* used the deformation of a square wave to measure the electric properties of the membrane, *Buchthal* uses an electrostatic estimation of the fibre potential with and without the fibre loaded with a suitable resistance.

If, however, the nerve fibre is impermeable to water, and, as was deduced above, impermeable to NaCl, the liberation of K^+ from the nerve during its activity cannot be accounted for, unless it is supposed, that the potassium is transported by some special mechanism influenced by electric stimulation, and by the injury poten-

tial. The theory of the fibre membrane acting as a filter cannot, therefore, be correct.

The supposition of a special mechanism, accounting for the potassium liberation during action is especially interesting when seen in the light of recent investigations by *Lundegårdh* [1940], and by *Krogh* [1943]. It is a well-known fact that certain vegetable cells have the ability of concentrating potassium, even against a high gradient. *Lundegårdh* measuring surface potentials of vegetable root fibres, investigated this fact, and built up a hypothesis as to how the ions were transported into the cell. According to this hypothesis the cell membrane is built up of long molecules, arranged with their axes perpendicular to the surface of the cell [cf. *Langmuir* [1939]]. These molecules have a special affinity to the ion concerned and are situated in such a way that they are able to turn round an axis parallel to the surface of the cell. Fig. 30.

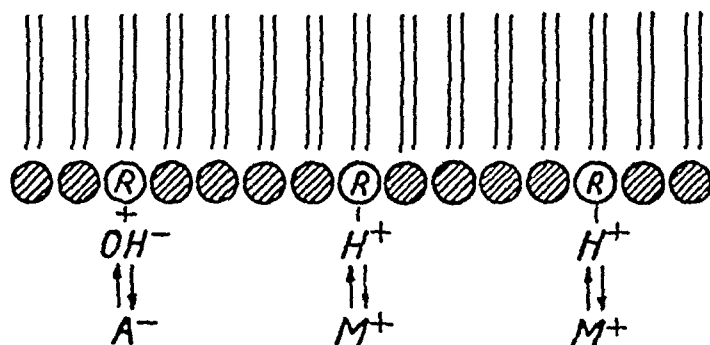


Fig. 30. *Lundegårdh's* Model of a Protoplasmic Surface (From *Lundegårdh*: Investigations as to the Absorption and Accumulation of Inorganic Ions. Ann. of the agricultural college of Sweden, 1940, 8, pag. 367).

Not all the molecules are supposed to be active in the absorption of ions. In the following diagrams we have only drawn the active molecules. The absorption of ions is supposed to be a balance-process.

If potassium ions are attached to the end of such a molecule on the surface of the cell, it will be transported into the inner surface when the molecule turns 180° .

The important fact is that this is a function of the living cell membrane, dependent upon various factors — ion concentration, temperature, pH, oxygen tension etc., and that it is independent of the permeability of the membrane in the old sense, meaning pore permeability. It can therefore only be investigated on the living

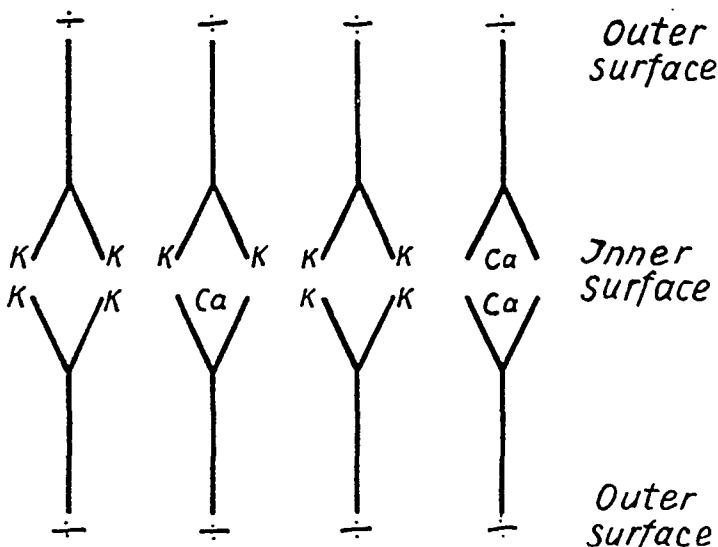


Fig. 31. Model Conception of Nerve Fibre Membrane. (Explanation see text).

This fig. and the figs 32, 34 and 35 only contain the molecules actively concerned with the ion transport while the "inactive" supporting elements indicated in fig. 30 are not included. Therefore the polarities $+$ and $-$, indicated in the fig., are not necessarily those of the membrane taken as a whole. The potential measured across the membrane is known to have its $-$ on the inside and its $+$ on the outside, this potential is composed of at least 4 main components: 1) The potential of the supporting elements (not indicated in the fig). 2) The potential of the ion-transporting elements. 3 and 4) The inner and outer surface potentials from the double layer on the inside and on the outside of the fibre respectively.

cell under "physiological" conditions and preferably by means of the radioactive isotopes as *Krogh* [1946] has pointed out, working with the same theory as *Lundegårdh* on muscle and other animal membranes.

A similar mechanism may be suggested for the nerve fibre membrane if the active part of this is composed of a mono-molecular layer of proteins or fatty acids.

A model conception is shown in Fig. 31.

This Fig. represents a longitudinal section through a fibre. The molecules concerned are dipolar and have a negative charge on the outer surface, while they are able to take up two potassium ions on the inner surface. Some molecules are attached to Ca^{++} instead of 2K^+ . The layer itself has a very low pore-permeability for water and, therefore, also for ions.

Under physiological conditions ions can only be transported

inwards, when the molecules turn, and in this way the fibre can be supposed to maintain its high internal potassium-concentration, as the molecules occasionally turn, when the nerve is at rest, transporting potassium through the membrane.

These "spontaneous" molecular movements are dependent upon the temperature, and the potassium loss at low temperature [Cowan [1934]] may be accounted for in this way.

The potassium transport through the nerve fibre membrane may be explained as above in terms of turning molecules. *Lundegårdh* however, in his paper [page 369] offers another theory according to which ions may be transported through protoplasm by "jumping along a molecular chain" — this may be an alternate explanation to the potassium transport into the axoplasm even though the theory is best supported by the experimental facts known at the present.

To return to the original hypothesis: if a fibre is stimulated electrically — current will pass through as shown in Fig. 32:

The fibre will first be charged like a condenser [*Hodgkin and Rushton* [1945]].

The same charging curve has been demonstrated on whole nerve by *Erlanger and Gasser*, 1937, page 143. The slope of the ascending

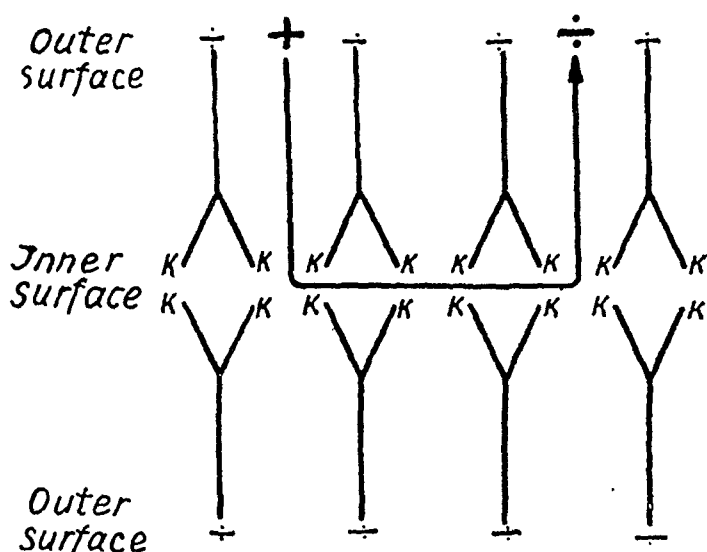


Fig. 32. Diagram of Electric Excitation. (Explanation see text). Stimulating current applied to the membrane (Conf. Fig. 31).

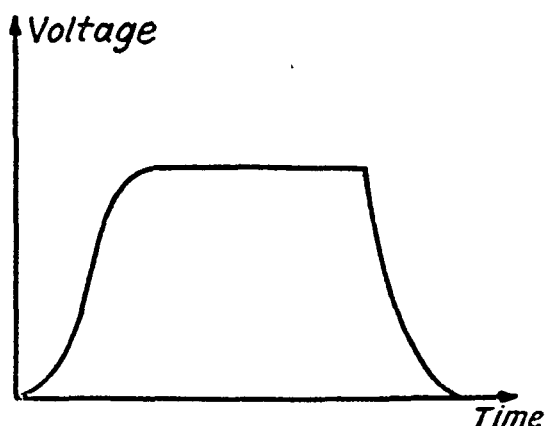


Fig. 33. Charging Curve of Nerve Fibre

Ordinate: Potential in arbitrary units.

Abcissa: Time in arbitrary units.

part of the charging curve, and the voltage reached will depend on [A] the voltage applied [B] the capacity of the nerve [higher for thick fibres with large inner and outer surface], [C] the resistance of the membrane.

When a critical voltage is reached, the inner stability of the mono-molecular membrane will be disturbed, and the molecules will tend to orientate in the direction of the current. The orientation of the molecules will be unchanged at the anode while at the cathode the negatively charged molecular end will be repulsed from the surface, and the end with the two potassium ions turned outwards. The converse will take place the moment the current through the fibre is discontinued, which corresponds with the facts of the positive and negative electrotonus [*Hermann* [1898], *Pflüger* [1899]].

A propagation theory may be based on these views —

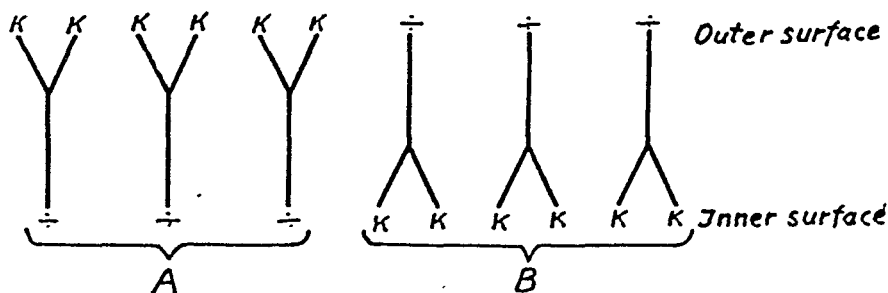


Fig. 34. Diagram of Impulse Propagation. Explanation see Text.

When the fibre is stimulated as described above, the position of the molecules in a section of the nerve membrane will be as Fig. 34.

The cathode applied at C has repulsed the negative charges of molecules A, and their potassium ions are turned towards the outer surface, while the position of molecules B remains unchanged.

The potassium ions of A will immediately be lost to the electrolyte solution round the nerve, which has a relatively low potassium content compared with that part of the fibre surface, where suddenly a high potassium concentration is achieved.

Molecules A, having lost their potassium, will generate a concentrated "focus" of negativity on the surface of the fibre.

When this negative potential reaches a certain critical level, it turns the adjoining molecules, and the impulse is conducted along the fibre; this travelling impulse is the action potential.

This corresponds with the work of *Hodgkin* [1937], in which he assumes that the impulse is in fact propagated by the action potential, and also with the findings of *Hodgkin* and *Huxley* in a recent work [1945]. They recorded action potentials from electrodes introduced into the giant fibres of the squid and found actual reversal of the membrane polarity during activity. A similar conclusion has been drawn by *Curtis* and *Cole* [1940—42].

When the impulse has passed, the molecules will take up potassium one by one and turn through 180° . During this process each molecule will transport a negative charge to the surface which may be the explanation of the negative after-potential.

Probably conduction takes place in segments [*Matteucci* [1840], see page 3], so that only one small part of the nerve fibre is in the excitatory state; this part excites the next segment, and so on. Evidence to this effect seems to have been brought forward by *Erlanger* and *Blair* [1934], and by *Tasaki* [1941], but it does not necessarily mean that the segments are anatomically defined.

If the number of active molecules in a segment is decreased, the segment will have to be extended in length to provide sufficient molecules with negative double charges for generation of the critical potential.

This means 1. That it will take longer to charge the prolonged segment to the critical potential.

2. The amplitude of the action potential will be smaller owing to greater leakage of current. The electrical leakage in the membrane will also be increased on account of the abnormal high permeability as well at a low temperature as in the osmotic damaged fibre.

3. Probably the whole potential, including the after-potential, will be prolonged.

These suggestions correspond to the results of our investigations, on fibres permanently damaged in an iso-osmotic solution [chapter 4] or reversibly damaged by low temperature [chapter 7]. In both cases it may be presumed that a certain percentage of the molecules are inactive owing to protein coagulation in the former, and the temperature fall in the latter. —

The investigations showed that:

1. The conduction rate diminished.
2. The amplitude was reduced.
3. The whole potential, and especially the after-potential, was prolonged.

It is noteworthy that thin fibres, such as sympathetic nerve fibres have slow conduction rates, small amplitudes, and action potentials of long duration.

According to this theory it is essential for the propagation of the impulse that the molecules lose the potassium ions concentrated on the outer surface. This takes place only when the fibre is surrounded by an electrolyte solution with low potassium content.

The dependence of the action potential upon the potassium concentration has been shown by *Cowan* [1934], and in this paper [chapter 5], while *Erlanger* [1938] showed that reversible blocking could be produced in fibres surrounded by watersoluble anelectrolytes.

The theory is in agreement with the effect of decalcination with citrate or oxalate, [*Lehmann* [1937], chapter 6]. Decalcination produces free negative charges at the inner end of the molecules normally containing Ca^{++} [Fig. 30].

Whenever a number of these molecules turn simultaneously, excitation takes place and spontaneous discharges will result.

The membrane system needs little energy to disturb it [mecha-

nical stimulation]. The reorganisation of the molecules is dependent upon energy from the chemical processes in the fibre, which liberate heat [*Hill's* recovery heat]. The process of reorganisation is more extended, when disorganisation has been complete. This will be the case where artificial electrical stimulation has been used. On account of the unsuitable wave form of the excitation current and loss to the surrounding fluid and in the membrane, the minimal electrical stimulus is always of higher amplitude than the action potential, hence the higher oxygen consumption of the excited part of the fibre compared with the conducting part [*Buchthal and Hertz* [1945] chapter 7].

It may be assumed that the reorganisation commences as soon as the molecules begin to turn. Therefore, if the excitation current is increased slowly, the reorganisation will keep up with the molecular movements, and the critical potential for propagation will never be reached. The laws of accommodation [*Hill* 1936] can be seen from this angle.

The movements of the molecules will have a certain resonance dependent upon their molecular weight and their position in the membrane, a fact which may account for the finding of an optimal A. C. frequency for stimulation [*Coppee* [1937], and *Arvanitaki* [1939]]. Finally it can be mentioned that the sensitivity of nerve fibres to stretching is explained by the supposition that the strain is taken by the monomolecular layer which is described as the functional element of the fibre.

The potassium transport through the surface of the active nerve fibre has been explained above, after the same principles as used by *Lundegårdh* [1940]. A membrane built up in this way, however, not only accounts for the selective potassium transport but is compatible with experimental results on the nerve impulse.

In spite of this agreement between the hypothesis and the experimental facts it remains to be proved that the membrane is built in the way described. For this purpose it will be necessary to provide some entirely new data.

The possibility of influencing the molecular movements by a strong magnetic or electrostatic field has been considered, but owing to the radial arrangement of the molecules in the fibre it is doubtful, if this will prove successful.

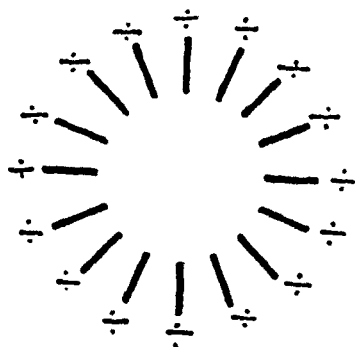


Fig. 35. Radial Arrangement of Molecules.

The radial arrangement may be the cause of the independence of the fibre of surrounding electrostatic fields. Although influence by one fibre on another has been proved to take place in a whole nerve, [*Arvanitaki* [1942]], the critical level for stimulation is not reached under physiological conditions. The molecular arrangement described will also prevent X-ray spectrographic and birefringence-methods from being of much help in investigations. As *Krogh* [1945] has pointed out, probably the only way to examine molecular movements of this sort is by the use of radioactive isotopes. In the use of the radioactive potassium isotope on nerve fibres two main difficulties will arise: 1. that the potassium liberation from ordinary medullated nerve fibres is probably too small to record — this may be overcome by the use of giant fibres from cephalopode, snail or earthworm — 2. that the potassium inside the fibre cannot be exchanged for radioactive potassium — this may be overcome by the storage of the fibre at a low temperature, under which condition it will lose some of its potassium. When the temperature is raised, radioactive potassium may be introduced instead.

If a fibre containing radioactive potassium is stimulated in a non-radioactive fluid, all radioactivity recorded from the fluid must be due to potassium liberated from the fibre. Comparison between the potassium liberation recorded in this way and changes in the action potential during exposure of the fibre to temperature changes and electrotonus may provide further evidence on the mode of action of the nerve fibre.

SUMMARY

THE *introduction* explains, why recording of action potentials is the method of choice for investigation of the nervous impulse and further why this investigation should be carried out on isolated nerve fibres. A summary is given of earlier investigations of the ionic content of the nerve and of the dependence of the action potential and demarcation potential upon the chemical composition of the fluid surrounding the nerve. We thought it useful to reinvestigate these problems on single fibres with a uniform electrical technique adjusted to modern claims [cathode ray oscillograph to give a true record of the development of the potential, resistance measurements between leading-off electrodes and a special arrangement to prevent stimulus escape from interfering with the records]. Further we propose to introduce diameter measurements on the single fibres as an indicator of their permeability.

Chapter 1 gives a short historical survey over the development of our knowledge about the mechanism of nervous action from *Galvani's* demonstration of muscular twitchings in 1786 until the present day.

Chapter 2 is an account of the technique used in this work. Part I describes the preparation of the isolated fibre and the method for measuring its diameter. The standard deviation and mean error of a diameter estimation are calculated as well as the physiological diameter of the various fibres. A special chamber for dissection and leading off from a nerve fibre in a moist atmosphere is described. The chamber has a separate excitation part, and the two parts may be separately filled with hydrogen. The temperature in the chamber may be controlled by leading

water of different temperature through a glass double bottom. Part II describes the electrical and photographic technique, the stimulators and the special mechanism to prevent stimulus escape, and to synchronize stimulus and sweep of the oscillograph. The four stage amplifier, the calibrating arrangement, the time-base generator, the bridge to measure the resistance between the leading-off electrodes, the camera, the measurement of the curves, are all described. Part III is an account of the chemical technique. The preparation of the various fluids, the control and checking of their ionic content, pH, oxygen tension and temperature are mentioned. Action potentials were recorded from a fibre, which was stimulated every minute during a period of 2-3 hours to ensure that the fluids used were adequate. — In an addendum is described the preparation of a colloid Na-permutite ion exchanger.

Chapter 3 is a detailed study of the action potential of a single nerve fibre, measured under physiological conditions with the technique used in this work. At 17° C and pH 7.3 the latency period + the conduction time [distance 13 mm] was 0.4 ms. The ascending phase of the spike had a duration of 0.9 ms. The descending phase of the spike + the negative after-potential lasted for 1.1 ms and the positive after-potential lasted for 4.2 ms. The maximal amplitude of the spike with an interelectrode resistance of 40.000 ohms and an interelectrode distance of ca. 35 mm was 75 μ V.

Chapter 4 gives the results of simultaneous diameter- and action potential-records from fibres immersed in hyper- or hypotonic solutions. Solutions made up to 7 times hypertonic with NaCl or glucose, or $\frac{1}{3}$ hypotonic, were without effect on either diameter or action potential in the first 5 min. of exposure, proving the extreme water impermeability of the nerve fibre membrane. Later the nerve shrinks or swells by 10 per cent, and the maximal alteration in diameter is reached after 10—12 min. During the next 20—30 min. the fibre diameter slowly returns to its original value, even though the fibre remains in the aniso-osmotic solution. The point of maximal alteration in diameter coincides with the first apparent changes in action potential: increased conduction time, decreased amplitude and prolonged negative after-potential. During the second phase, where the diameter returns to its original value, the negative after-potential becomes progressively

slower until after 30—40 min. negativity persists indefinitely after a stimulus. The osmotic damage is only reversible, if duration of exposure is less than 5 min.

Chapter 5 is a further investigation of the blocking of the nerve impulse by potassium ions as described by *Cowan* and others. Immersion of the nerve fibre in iso-osmotic solutions with a KCl content of 1—10 times the normal, causes an immediate and reversible reduction in the amplitude of the action potential, while its shape remains unaltered. The reduction of the amplitude is dependent upon the potassium concentration. The potential is generally extinguished at a concentration about 7—8 times the "physiological" concentration [in our work = 2.66 millimol KC]. The effect of KCl can be only partially counteracted by an equivalent increase in the CaCl_2 concentration. An attempt to remove K^+ from the nerve by Na^+ -permutite exchanger was without effect. — K^+ concentrations below $10 \times$ the physiological value had no effect on the nerve fibre diameter. Concentrations about 10 — $15 \times$ the physiological caused even in iso-osmotic solutions a transient swelling of the fibre by 10 per cent. The action potentials were irreversibly extinguished in these strong K^+ concentrations after 3—5 min.

Chapter 6. I. Removal of Ca^{++} by citrate or oxalate caused spontaneous activity in the fibre, but not until 45 min. had elapsed. Exposure of the fibre to 10 times the physiological concentration of CaCl_2 [in our work = 1 millimol CaCl_2] for 20—30 min. caused a reduction of the spike amplitude and an increase of the amplitude of the negative after-potential.

II. MgSO_4 in concentrations 8.3—66.4 millimol were without any effect on the fibre action potential over several hours, while the same solutions had a paralysing effect on the motor endplate after 30—40 min.

III. Even drastic pH alterations, pH 5 — pH 9 were without effect on either action potential or potassium sensitivity of an isolated nerve fibre within 15—20 min. With longer exposure to alkaline solutions spontaneous discharges appeared as described on fibre bundles by *Lehmann*.

IV. Acetylcholine chloride was without any effect on the nerve fibre action potential in concentrations 1 μg — 0.01 μg per ml which caused spontaneous activity in bundles of muscle fibres.

Chapter 7 deals with the nerve fibre reaction to anoxia. When anoxia is applied to both the stimulated and the conducting region of a nerve fibre, the average time necessary to suppress activity is 34 min., while it takes more than 70 min., when anoxia is limited to the leading-off section of the chamber. The action potential reappears immediately after readmission of oxygen with reduced amplitude and without a positive after-potential. After 15 min. in atmospheric air its original shape and amplitude is regained. The stimulated region of a peripheral nerve fibre is thus far more sensitive to anoxia than its purely conducting parts.

Chapter 8 is a description of the temperature dependence of the action potential of a nerve fibre. While the ascending phase of the spike is almost independent of the temperature, the temperature dependence of the spike amplitude and of the conduction rate are somewhat greater. The after-potentials have, however, a temperature dependence of a much higher order than any other part of the action potential.

Chapter 9 gives a discussion of the results attained and a theory about the mechanism of nervous conduction. The impermeability of the intact nerve fibre to water and NaCl is shown in chapter 4, while *Cowan* has shown, that potassium is liberated from the fibre during conduction, therefore the potassium must be transported out of the fibre by some special mechanism. *Lundegårdh* suggests a theory explaining the iontransport into vegetable cells. According to his theory the cell membrane is built of long molecules, arranged with their axis perpendicular to the surface of the cell and able to take up the ion concerned by absorption at one end and transport it into the cell. This transportation may take place either by turning of the long molecule 180° , or by altering the charges at the ends of the molecule in such a way, that the ion will be attracted to the other end. This is a function of the living cell surface. A theory on those lines for the nerve fibre is able not only to explain the selective potassium transport through the surface of the fibre but also to give a picture of the impulse propagation. The data on nervous conduction provided from this investigation and those collected from literature correspond with this synopsis.

Finally, possibilities are discussed concerning the collection of further evidence on the theory.

RESUMÉ

Det foreliggende Arbejde er en Undersøgelse af Nerveimpulsens Afhængighed af de fysisk-kemiske Forhold i det Milieu, der omgiver Nervetraaden. Undersøgelsen er udført som et Forsøg paa at finde frem til en klarere Opfattelse af Mekanismen ved den Iontransport gennem Nervetraadens Overflade, der betinger Nerveimpulsens Opstaaen og Udbredelse.

I *Indledningen* motiveres det at anvende Registrering af Aktionspotentialer som et adækvat Udtryk for Nerveimpulsen; det omtales videre, at en saadan Registrering maa finde Sted paa isolerede Nervetraade for virkelig at faa et Udtryk for det enkelte Nerveelements Funktion. Forskellige tidligere Undersøgelser over Aktions- og Demarkationspotentialernes Afhængighed af Sammensætningen af Vædsken omkring Nerven nævnes. Naar vi har fundet Anledning til at genoptage disse Undersøgelser, skyldes det flere Aarsager: 1) De forskellige Faktorer Indflydelse paa Aktionspotentialer har været undersøgt af hver sin Forfatter, saaledes at en ensartet Teknik ikke har været anvendt, hvad der gør Resultaterne vanskelige at sammenligne med hinanden. 2) De tidligere Undersøgelser har været udført paa hele Nerver og ikke paa isolerede Fibre. 3) Den elektriske Teknik staar i de fleste af de tidligere Undersøgelser ikke Maal med moderne Krav. Det er derfor vor Hensigt at gennemføre Aktionsstrømsmaalinger paa isolerede perifere motoriske Nervetraade under Ændring af alle de fysiske og kemiske Faktorer i det omgivende Milieu, som direkte kan antages at influere paa Nerveimpulsens normale Udbredning. Til Registrering af Potentialerne har vi brugt Katodestraaleoscillograf for at faa et sandt Billede af Potentialets tidsmæssige Udvikling. Shuntmodstanden mellem Aflednings elektro-

derne har været underkastet Kontrol med Maalebro for at kunne vurdere de maalte Potentialers Amplitude. En særlig Anordning har været anvendt for at forhindre Irritationsindbrud, og endelig har vi indført Diametermaaling paa den enkelte Nervetraad under forskellige Forsøgsbetingelser for at faa et Begreb om Nerve-traadsmembranens Permeabilitet.

Kapitel 1 er en kort Oversigt over tidligere Arbejder paa dette Omraade fra *Galvanis* berømte Demonstration i 1786 til Nutiden.

I *Kapitel 2* omtales den anvendte Teknik. Første Del beskriver Præparationen af den isolerede Nervetraad. Et særligt, fugtigt Kammer omtales, i hvilket den hele Nerve anbringes til Mikrodissektion, og hvori den isolerede Nervetraad forbliver under Forsøgene. Kammeret har en regulerbar Udspændingsanordning for Nerven, og er forsynet med Irritations- og Afledningselektroder. Irritations- og Afledningsdelene er adskilte og kan hver for sig fyldes med Brint eller Ilt. Ved at gennemstrømme en Dobbeltbund med Vand af forskellig Temperatur kan Temperaturen i Kammeret reguleres. Bunden er af Glas for at muliggøre Belysning til Diametermaalingerne. Nervetraadenes fysiologiske Diameter angives, saavel som Middelfejl og Standardafvigelser paa den enkelte Diametermaaling.

Anden Del beskriver den elektriske og fotografiske Teknik, omfattende en 4-Trins Forstærker med Differentialindgang og Kalibreringsanordning, Kondensatorudladnings-Irritationsapparat, Anordning til at forhindre Irritationsindbrud og til at synkronisere Irritationen med Oscillografens Kip, Vekselstrømsmaalebro til at maale Modstanden mellem Afledningselektroderne, Tonegenerator til Tidsmarkering og til at levere Vekselstrøm til Maalebroen samt Kathodestraaleoscillograf og Kamera. Endelig beskrives Maalingen af de optagne Kurver.

I tredje Del gives en Fremstilling af de anvendte Vædske's Tilberedning og Kontrollen med deres Ionindhold, pH, Iltspænding og Temperatur. For at konstatere om den anvendte Normalvædske er adækvat, registreres et Aktionspotential fra en Nervetraad umiddelbart efter Dissektionen, samt efter at Traaden i 2—3 Timer er blevet stimuleret hvert Minut; Aktionspotentialet holder sig uforandret. I et Tillæg beskrives Tilberedningen af en kolloid Na-Permutit-Ion-bytter.

Kapitel 3 er en Beskrivelse af en isoleret Nervetraads Aktionspotential, maalt paa den angivne Maade under fysiologiske Forhold. Den isolerede motoriske Traads Aktionspotential bestaar som hele Nervens af et spike potential samt et negativt og et positivt Efterpotential. Varigheden af Spikepotentialets opadstigende Del er 0,9 ms-Varigheden af dens nedadstigende Del + det negative Efterpotential er 1,1 ms, endelig varer det positive Efterpotential 4,2 ms. Spikepotentialet naar en gennemsnitlig Amplitude af $75 \mu V$, maalt med en Interelektrodemodstand paa ca. 40.000 Ohm og en Afstand mellem Afledningselektroderne paa ca. 35 mm.

Kapitel 4 giver Resultaterne af samtidige Aktionspotential- og Diametermaalinge paa Nervetraade i forskellige hyper- og hypotoniske Opløsninger. Det viser sig, at Nervetraadens Diameter først paavirkes af stærkt anisoosmotiske Opløsninger. Der skal $7 \times$ den normale osmotiske Koncentration for at faa Traaden til at skrumpe maaleligt, og Opløsningen skal ned paa $\frac{1}{3}$ af den isoosmotiske Koncentration for at faa Traaden til at svulme. Selv i disse stærkt anioosmotiske Opløsninger sker Diameterændringerne først efter en Latenstid paa ca. 5 Minutter, Ændringerne udgør ca. 10 % af Traadens oprindelige Diameter, og den naar sit Maximum efter ca. 8—9 Minutters Forløb; Diameteren vender spontant tilbage til sin Udgangsværdi efter 20—30 Minutter, selv om Nervetraaden forbliver i den hyper- eller hypotoniske Vædske, og Nervefibren er da ikke mere følsom for Forandring i den osmotiske Spænding. Skrumningen af Nervetraaden er upaavirket af, om Vædsken gøres hypertonisk med NaCl eller med Glukose. Aktionspotentialet er temmelig upaavirket, indtil Diameteren naar sit Maximum eller Minimum; paa dette Tidspunkt sker der en Formindskelse af spike-Amplituden, men det mest markante Udslag er en stærk Forlængelse af det negative Efterpotential. Denne Forlængelse af Efterpotentialet tiltager stadig, og naar Nervetraadens Diameter har naaet sit Udgangspunkt, vedbliver Negativiteten at bestaa længe efter en Irritation. — De beskrevne, af Ændringer i det osmotiske Milieu betingede, Forandringer i Nervetraadens Diameter og Aktionspotential er alle irreversible. — Af de stærkt anisoosmotiske Opløsninger, der er nødvendige for at fremkalde Diameterforandringer, og af den lange Latenstid drages den Slutning, at *Nervetraadsmembranen*

er meget lidt permeabel for Vand. Den spontane Tilbagegang og de irreversible Ændringer i Aktionspotentialet tyder paa, at Membranen er beskadiget.

I *Kapitel 5* undersøges den af Kalium fremkaldte Blokering af Nerveimpulsen. Fjernelse af Kalium fra Vædsken omkring Nerve-
traaden eller Forøgelse af Kaliumindholdet med $1-2 \times$ den fysiologiske Koncentration fremkalder ingen Forandringer i Aktionspotentialet. Forøges Kaliumindholdet med $3-7 \times$ den fysiologiske Koncentration, sker der en øjeblikkelig reversibel Reduktion af Aktionspotentialets Amplitude, uden at Potentialets Form ændres. Selv paa enkelte Nervetraade er Amplitudereduktionen gradvis afhængig af Kaliumkoncentrationen. Afhængigheden er *ikke* logarithmisk, som man skulde vente det, hvis der var Tale om en ren Koncentrationseffekt. Forøges Kalciumkoncentrationen proportionalt med Kaliumkoncentrationen, modvirkes Amplitudereduktionen delvis. K^+ kunde ikke fjernes fra Nerven med Na-Permutit Ion-bytter. Forøgelse af Kaliumkoncentrationen omkring Nerve-
traaden indtil $7-8 \times$ den fysiologiske Koncentration fremkalder ingen Ændringer i Nervetraadens Diameter, forøges Koncentrationen derimod $10-15 \times$ den fysiologiske, tiltager Diameteren ca. 10 % i Løbet af 10—12 Min. og vender derpaa spontant tilbage til sit Udgangspunkt i Løbet af 20—30 Min. I disse stærke Kaliumopløsninger er Aktionspotentialet irreversibelt udslukt efter 3—4 Min. Alle Kaliumforsøg er foretaget under isoosmotiske Forhold.

Kapitel 6. I. Fjernelse af Kalcium med Citrat eller Oxalat forårsager spontan Aktivitet i Nervetraaden, men først efter 45 Minutters Forløb. Paavirkning af Traaden med 10 Gange den fysiologiske Koncentration af Kalcium (i vort Arbejde ≈ 1.8 millimol) i 20—30 Min. forårsagede en Reduktion af Spikepotentialets Amplitude og en Forøgelse af det negative Efterpotentials Amplitude.

II. Magniumsulfat er uden Effekt paa Nervetraadens Aktionspotential i Koncentrationer paa 8.3—66.4 millimol, selv naar Paavirkningen varer adskillige Timer, medens de samme Opløsninger har en lammende Effekt paa den motoriske Endeplade efter 30—40 Minutter.

III. Selv drastiske pH-Ændringer, pH 5—pH 9, er uden Virkning baade paa Aktionspotential et og paa dettes Afhængighed af Kaliumkoncentrationen i de første 15—20 Minutter. Ved længere Paavirkning af Nervetraaden med alkaliske Opløsninger optræder spontane Udladninger, som beskrevet paa hele Nerver af *Lehmann*.

IV. Acetylchlinklorid er uden Indvirkning paa Nervetraadens Aktionspotential i Koncentrationer fra 1 μg pr. ml til 0.01 μg pr. ml. Alle disse Opløsninger fremkalder Aktivitet i Muskelfiberbunder.

Kapitel 7 omhandler Nervetraadens Reaktion paa Anoxi. Udsættes baade den stimulerede og den ledende Del af en Nerve traad for Anoxi, varer det gennemsnitlig 34 Minutter at udsukke Aktionspotential et, men hvis Anoxien kun paavirker den Del af Nervetraaden, der leder Impulsen, og hvorfra Aktionspotential et afledes, tager det ca. 70 Minutter, før Potential et udslukkes. Aktionspotential et kommer igen umiddelbart efter at Nervetraaden atter bringes i Iltatmosfære, men med en reduceret Amplitude og uden positivt Efterpotential, efter 15 Minutter i atmosfærisk Luft har Potential et genvundet sin oprindelige Amplitude og Form. Irritabiliteten af en perifer Nervetraad er saaledes meget mere følsom for Iltmangel end Impulsledningen.

Kapitel 8 er en Beskrivelse af Aktionspotential ets Afhængighed af Temperaturen. Medens Varigheden af spike-Potential ets opadstigende Del er proportionalt med den absolute Temperatur, er Temperaturafhængigheden af spike-Amplituden og Ledningshastigheden noget større. Efterpotentialerne har imidlertid en Temperaturafhængighed af en meget højere Størrelsesorden end nogen anden Del af Aktionspotential et.

Kapitel 9 diskuterer dette Arbejdes Resultater og formulerer en Teori om Nerveledningen. Da det er vist i Kapitel 4, at Nerve traaden er meget impermeabel for Vand og Natriumklorid, medens *Cowan* har vist, at Kalium frigøres fra Nervetraaden under Impulspassagen, maa denne Kaliumtransport gennem Nervetraadens Overflade forklares ved en speciel Mekanisme. Iontransport gennem Protoplasmaoverflader er blevet undersøgt af *Lundegårdh*, der arbejder med Transporten af Ioner ind i og ud af Planteceller. Efter en Teori formuleret af *Lundegårdh* er Cellemembranen opbygget af lange, elektrisk ladede Molekyler, der tænkes anbragt

med Længdeaksen vinkelret paa Celleoverfladen, og som er i Stand til at optage den paagældende Ion ved Absorption i den ene Ende og transportere den ind i Cellen enten ved at Molekylet drejer 180° eller ved at Molekylets Ladning ændres paa en saadan Maade ved Absorptionen, at den paagældende Ion af elektriske Kræfter drives til Molekylets anden Ende. Denne Ionpermeabilitet er en Funktion af den levende Protoplasmaoverflade. Hvis *Lundegårdhs* Teori benyttes paa Nervetraaden, er den i Stand til ikke alene at forklare den selektive Kaliumtransport gennem Nervetraadens Overflade, men det er muligt, paa Basis af den, at udforme en Nerveledningsteori. En saadan Nerveledningsteori passer ind med alle Data om Nerveledningen, der er fundet i dette Arbejde, saa vel som med en hel Del vigtige Data, samlede fra Litteraturen.

Til Slut undersøges Mulighederne for at samle yderligere Materiale til Støtte for Teorien.

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STUDIES ON THE INTERRELATION OF THE
SULFONAMIDES AND THE THIAMINE BALANCE
OF THE ORGANISM, AND ON THE ACETYLTATION
OF THE SULFONAMIDES

BY
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PREFACE.

The present work has been carried out in the Department of Medical Chemistry, University of Helsinki, during the years 1944—1946.

To Professor P. E. Simola, Head of the Institute, I owe my deepest gratitude for the present theme. His neverfailing interest and advice have been of greatest value for the investigation, and the accomplishment of the work was made possible only by his kindness in placing the laboratory with the necessary equipment at my disposal. — In this connection I wish also to thank the members of the staff of the Institute, whose assistance and professional skill I have been permitted to take advantage of. — I am likewise greatly indebted to V. M. Nurmia, Ph. D. for his translation of the present work from Finnish into English.

The lack of Anglo-Saxon literature, caused by our involuntary isolation during the recent years, has impeded all medical research work in this country. This condition is making itself

sorely felt also in the scientific literature dealing with the present theme. Thus, I have been compelled to some extent to make use of referating literature.

I wish herewith to express my sincere thanks to the Finnish Culture Fund for the scholarship granted in support of this investigation.

Helsinki, in October, 1946.

O l a v i K i n n u n e n.

INTRODUCTION.

During the decade which followed the discovery of the sulfonamides, the use of sulfanilamide — the active component of prontosil — and its innumerable derivatives has benefited greatly most branches of medicine. Side by side with increasing clinical experience, research has been extended also to the various metabolic and toxic properties which the sulfonamides were found to possess. The number of papers dealing with the clinical use and toxic properties of the sulfonamides has swelled up to several thousands, whilst comparatively little work has been done on their physiologic properties. However, the clinical use of the sulfonamides is apparently closely associated with certain central phases of general physiology, the metabolism of vitamin B₁ and the problem of acetylation. The present work was undertaken to shed some light on these questions.

For a better understanding of the work it is necessary to be acquainted with certain properties of the sulfonamides. A description of the physiology of the sulfonamides and their fate in organism is therefore included, although some of these facts can be expected to be generally known.

Part I

The physiologic properties of the sulfonamides.

Absorption of sulfonamides.

Sulfonamides may be administered *per os*, *per rectum*, and as subcutaneous, intravenous, intramuscular and intralumbar injections, or they may be injected directly into the body cavities (pleural, abdominal and joint cavities). They can be applied in powder form on mucous membranes and on lacerated and wound surfaces, and directly on the skin in the form of pastes and suspensions.

When sulfonamides are given *per os*, there is a partial absorption already in the stomach. Most of the absorption occurs in the small intestine and only very little in the large intestine (*e.g.* STEAD and KUNKEL, 1940), so that absorption is practically complete already before the ileocecal region is reached. Sulfanilamide, sulfathiazole and sulfamethyl thiazole are most rapidly absorbed, while with sulfaguanidine, succinyl sulfathiazole and phthalyl sulfathiazole the absorption is slower (*e.g.* HAWKING, 1942; FRISK, 1943; ELDAHL, 1943, 3). The latter compounds have therefore been held particularly suitable in the treatment of affections of the large intestine and in the preparation of the bowel for operation (*e.g.* POTH, 1945). The irregular resorption of sulfapyridine is well known.

If sulfonamides are taken by mouth on an empty stomach they, like other remedies in general, are more quickly absorbed, but the danger of nausea increases. Since sodium carbonate diminishes the precipitation of the sulfonamides in the stomach, administration of soda promotes resorption, at the same time counteracting the development of kidney complications (BARLOW *et al.*, 1941). WILSON (1943), however, maintains that carbonates have no significant effect on resorption. Higher blood concentrations of the sulfonamides are obtained also with urea (SOBIN *et al.*, 1943; FRISK, 1944). The rate of resorption is reported to be increased by vitamin B₁ and retarded by vitamin C; in both cases, however, the resorp-

tion is more complete (RARABEK, 1943). The sodium salts of the sulfonamides, being generally easily soluble, are resorbed more quickly than the free sulfonamides, in spite of the fact that they are precipitated to a large extent in a normally acid stomach (SOBIN, 1942).

When sulfonamides are given by the tube directly into the duodenum, absorption of the less-soluble drugs diminishes, while with more easily-soluble sulfonamides the effect of the duodenum is less. Resorption from the large intestine is slow if the drugs are injected as high *clysters* (e.g. STEAD and KUNKEL, 1940; TURELL *et al.*, 1940). When sulfonamides are given *per rectum*, variable blood level values are obtained (TURELL). Sulfapyridine and sulfadiazine are poorly resorbed if given *per rectum* (e.g. REINHOLD *et al.*, 1940). The sulfonamides are resorbed more quickly *per rectum* in the form of sodium salts (ANDERSEN, FRIEDBERG *et al.*, 1942).

Certain compounds — like sulfathiazole and sulfamethyl thiazole — which are resorbed at a similar rate when given orally, may produce altogether different blood levels when given as injections (ELDAHL, 1943, 2, 3). In spite of the solubility and quick resorption of the sodium salts it is nevertheless better to use *intramuscular* or *intravenous* injections when a rapid effect is required. In view of possible toxic complications it is not advisable to use *intralumbal* injections even in cases of meningitis.

When applied in the form of *wound powder*, sulfanilamide is absorbed best, then comes sulfathiazole (e.g. GOODWIN *et al.*, 1941; HAWKING and HUNT, 1942; HODGSON *et al.*, 1942). According to HAWKING, it is therefore best to apply to wounds a mixture of 2—3 parts sulfanilamide and 1 part sulfathiazole.

In *paste form* the sulfonamides are poorly resorbed into a healthy skin and the blood level values obtained are insufficient from the viewpoint of general therapy (STRAKOSCH *et al.*, 1943; CORHRAN, 1944; GARAZSI, 1944). The resorption is increased by oil and alcoholic vehicles (ANGEVINE, 1943; DREAGER *et al.*, 1945). When aqueous vehicles are used, the resorption from *burns* is so rapid that the concentration in the blood may reach toxic values. Oil vehicles, on the other hand, are safe (EVANS *et al.*, 1945).

Intraperitoneal use of the sulfonamides in connection with surgical operations is generally practised; in fact, resorption through the intact peritoneum is relatively rapid. Sulfonamides have also been given *intra-abdominally* through the peritoneum (ZACHO, 1945). Resorption from the *pleura cavity* is irregular and depends on individual and pathologic-anatomic factors.

Distribution of sulfonamides in the organism.

The concentration of the sulfonamides in the blood depends on the compound and dosage, but is independent of the amount of fluid administered (ALYEA *et al.*, 1939). The form of the concentration curve with oral intake depends on resorption. With a single dose of 4 g to a healthy person, sulfa-

nilamide, sulfathiazole, sulfapyridine and sulfaguanidine give maximum values after 1, 4, 6, and 6—8 hrs, respectively (FRISK, 1943). The accompanying table — compiled chiefly according to FRISK — shows some maximum blood values obtained with single and continuous doses.

Compound	Maximum values after a single dose of 4 g, mg-%		Values obtained after 40 hrs with continuous dosage of 2 g every 4 hrs, mg-%	
	free	total	free	total
Sulfanilamide	7 —10	8 —11	6	8
Sulfaguanidine	1 —2.5	1.5—3	2	3
Sulfathiazole	5 —7	6 —7.5	4	5.5
Sulfapyridine	3.5—5	4 —5	5	7

Intravenous and intramuscular injections raise the blood concentration of the sulfonamides more quickly and to a higher level.

If sulfonamide solution is added to the plasma and the mixture is deproteinised, the loss of sulfonamide on precipitation varies with the compound. Also under physiologic conditions, part of the sulfonamide is bound to the blood protein which, according to the cataphoresis experiments of SCHÖNHOLZER (1940), is the albumin.

Of sulfanilamide, 10—12 % is bound, and of sulfathiazole 75—80 %, the values for other sulfonamides varying between these limits. The combination of free and acetylated sulfonamides follows the same rules. In the estimation of sulfonamides, DRUEY and OESTERHELD (1942) recommend a heating of the sample on the waterbath for a couple of minutes after addition of trichloroacetic acid as a means of liberating the protein-bound part of compound. Protein-bound sulfonamide is bacteriostatically inactive but forms a potential reserve of the drug in the blood. The diffusion of the drug into the erythrocytes and the cerebrospinal fluid depends in part on the combination with protein.

After absorption into the blood stream — whether given orally or *per rectum*, injected intramuscularly or intravenously, or applied to wounds or mucous membranes — *sulfonamides diffuse approximately evenly in the tissues*. When 0.1 g of sulfanilamide, sulfapyridine or sulfathiazole is given orally by the tube to full-grown rats, the sulfonamide concentration in the tissues rises continuously during the first 3—4 hours after the single dose, remaining, however, lower than the concentration in the blood. The sulfonamide concentrations in the tissues and in the blood reach a maximum 4—5 hours after the dose. Afterwards the concentration in the blood falls more rapidly than that in the tissues so that after 8—10 hours the tissues show a higher value than the blood. The sulfonamide concentration seems generally to be slightly higher in the liver and kidney than in other tissues. Particularly when large doses of some easily-acetylated compound (like sulfapyridine) are used, high kidney values will be obtained, due to

disturbances in excretion. The ratio of free to acetylated sulfonamide is approximately equal in the tissues and blood. For obvious reasons, the kidney often contains more of the acetylated form. The total values in the fat tissue often remain low but, owing to slow elimination, this tissue is the last to contain the drug (*i.a.* own unpublished data).

JANBON *et al.* (1942) have studied the distribution of the sulfonamides in the organs of patients dying of meningitis and found high concentrations in the liver, spleen, prothata, pancreas, kidney, testis and adrenal glands, while low figures were obtained for the lungs, intestines, fat, bone, skin and peripheric nerves. BECHER-CHRISTENSEN and SIMESEN (1944) determined the sulfonamide content in the processus mastoideus and concluded that poor therapeutic results were not due to insufficient concentrations. They have also (1941) examined the tonsils of tonsillectomia patients and found that, of the sulfonamides tested, sulfanilamide showed the most rapid penetration into the tonsils.

The distribution of the sulfonamides between the plasma and the erythrocytes is an important factor in view of certain bloodtoxic complications. It has been found that those sulfonamides which penetrate most rapidly into the blood cells, also contribute to anaemia and cyanosis. For instance, sulfanilamide and sulfaguanidine are found more in the erythrocytes, while the plasma contains more sulfapyridine, sulfathiazole, sulfamethyl thiazole, sulfadiazine and sulfamerazine *etc.* (*e.g.* FRISK, 1941, 2, 1943; SIMESEN, 1941). The sulfonamides do not penetrate through blood coagulum, for instance, in cases of endocarditis (DUNCAN and FAULKNER, 1940).

Penetration into the cerebrospinal fluid has some importance in the therapy and, according to some authors, also in the diagnosis. From the viewpoint of therapy it is to be noted that, in all conditions, sulfathiazole, sulfamethyl thiazole and sulfaguanidine penetrate incompletely into the liquor (only 10—30 % of the concentration in the blood). Due to its small molecule, sulfanilamide reaches almost equal concentrations in the liquor and in the blood, while the values for sulfapyridine and sulfadiazine in the liquor are somewhat lower (50—90 % of concentration in the blood). Prontosil soluble and uliron do not penetrate at all into the liquor. The therapeutic effect, for instance in meningitis, depends largely on the concentration of the sulfonamides in the cerebrospinal fluid. Intralumbal administration has been considered unnecessary since a sufficient concentration of most compounds is reached in the liquor by other, safe means (*e.g.* VONKENNEL *et al.*, 1939; SÄKER, 1939; BECHGAARD *et al.*, 1941; KATZENELBOGEN, 1941; FRISK, 1941, 1; ROELSEN and SIMESEN, 1942). ANDERSEN and SIMESEN (1943) found that particularly sulfathiazole diffuses more easily into the liquor in cases of tuberculous meningitis. BECHGAARD *et al.* (1941) and HERBERTS (1944) likewise noted that meningitis alters the diffusion of the sulfonamides into the cerebrospinal fluid. — The elimination of the sulfonamides from the liquor is slower than from the blood (*cf.* other tissues).

With the ordinary dosage, concentrations equalling that in the blood will be reached in the *oedema fluid* (BECK, 1939) *peritoneal* and *pleura exsudates* and *pericardium fluid* (BARLOW and CLIMENKO, 1941; REINHOLD *et al.*, 1941; FRISK, 1941, 1) while in *pemphigus vesicles* the concentration may be still higher (NILZEN, 1945). The dye-stuff of prontosil does not diffuse from the blood into the pleura cavity but does so from the pleura cavity into the blood (BAUER, 1940).

When sulfonamides are given orally they can be detected 15 mins later in the *aqueous humour*, where a maximum concentration is reached already in 6 hours. There seem to be slight differences in the concentrations in different parts of the eye. Sulfathiazole diffuses into the eye more poorly than the other sulfonamides (BELLOWS and CHINN, 1939; MEYER *et al.*, 1941; ROBSON and TEBRICH, 1941). When sodium salts of the sulfonamides are applied locally in powder form, the concentrations obtained in the eye will be 3—4 times greater than when free sulfonamides are employed (P'AN, 1941). When iontophoresis is used in animal experiments, it is possible to obtain in the fore part of the eye higher concentrations of sulfonamides than otherwise (v. SALLMAN, 1942).

Sulfonamides pass easily through the *placenta*. In general, the ratio of the sulphonamide concentrations in the foetal and maternal blood is from 6/10 to 8/10, independently of the mode of dosage (SPEERT, 1938; PHLIPP, 1941; ANDERSEN and SIMESSEN, 1942).

The ratio of free to acetylated sulfonamide in the *foetus* is generally the same as in the mother; in some cases, the foetal blood contains less of the acetylated form (SPEERT, 1938). The azo dye-stuff of prontosil rubrum does not diffuse through the placenta (KAYSER, 1941). The sulfonamide concentration in the placenta is equal to that in maternal blood (ANDERSEN and SIMESSEN, 1942); the concentration in the *liquor amnii* corresponds to that in the foetal blood (STEWART and PRATT, 1938).

According to SPEERT (1942, 1943) the sulfonamides are resorbed poorly during the *later stages of pregnancy* and particularly during the labour. Consequently it is recommended to give to the mother large intravenous doses of sulfonamides in cases when it is desired to treat effectively infections in the mother or to protect the child against infection by the gonococci *etc.* Such dosage has not been found to be harmful to the child.

Metabolism of sulfonamides.

Of the changes which the sulfonamides undergo in the organism, acetylation is probably the most important and best studied one. Since the major part of the present work is closely associated with acetylation, this process will be dealt with in a separate chapter later on (p. 42).

There are also other possible ways by which the sulfonamides may be metabolised in the organism. One such possible way is the formation of *p-hydroxy-amino acid derivatives*. The formation of this acid from sul-

fanilamide, sulfapyridine and sulfathiazole was considered possible by SHELSWELL and WILLIAMS (1940), RAVINA and MAIGNAN (1940), THORPE and WILLIAMS (1940), JAMES (1940, 2), THORPE, WILLIAMS and SHELSWELL (1941) and SCUDI and ROBINSON (1941). This process has also been associated with the formation of acetyl derivatives. SCUDI (1944) has later isolated hydroxyaminosulfapyridine. In view of the potency of *p*-hydroxy-sulfonamide both *in vivo* and *in vitro*, this compound has been regarded as a possible precursor to the active form of the sulfonamide.

This assumption seems very improbable, however, considering our present views of the mode of action of the sulfonamides and of *p*-aminobenzoic acid. In fact, the work of JULIUS and VINKLER (1941) seems to indicate that it is not necessary for the sulfonamide molecule to be converted into another form to become effective.

In their work on the porphyrinuria in rats, caused by sulfanilamide, RIMINGTON and HEMMINGS (1939) showed that part of the sulfanilamide is oxidised. They ascribe to this oxidised form not only porphyrinuria, but also certain other toxic complications. By treating sulfanilamide and sulfapyridine with H_2O_2 *in vitro*, JAMES (1940, 1) obtained a number of oxidation products which he assumes would be formed also *in vivo*. In another work he was unable, however, to demonstrate in the urine, after administration of sulfapyridine, components other than the free and acetylated forms. FOX (1940) likewise assumes that the sulfonamides undergo oxidation in the organism.

Other possible ways of conversion of sulfonamides are the formation of 1-amino-2-hydroxybenzene-4-sulfonamide, 2-oxybenzoxazole-6-sulfonamide, 1-amino-3-hydroxybenzene-4-sulfonamide (THORPE and WILLIAMS, 1940; THORPE, WILLIAMS and SHELSWELL, 1941) and pyrochatechol sulfonamide (SAMMON, SHELSWELL and WILLIAMS, 1941).

In addition to the above modes of conversion, it is also possible that sulfonamides are conjugated with other components. In the first place *they may be detoxicated by conjugating with glucuronic acid or, etherally, with sulfuric acid, to form coupled acid compounds which are excreted with the urine.* The formation of coupled glucuronates or sulfates is a well-known process by which the organism detoxicates foreign and harmful compounds.

This process is a two-phase reaction, the first phase being the conversion of the sulfonamide to a phenol-like compound and the second its conjugation with glucuronic or sulfuric acid by means of the hydroxyl group. SHELSWELL and WILLIAMS (1940) have shown that if sulfanilamide is fed to rats or rabbits, part of the *p*-aminophenol and *p*-hydroxy-aminobenzoic acid is excreted etherally combined with sulfuric acid whose concentration in the urine increases. If we assume that one molecule of sulfanilamide yields one molecule of these phenols, 6–12% of the sulfanilamide is oxidised to phenol. The said authors were unable to obtain

similar results with sulfapyridine. These findings are in agreement with the observations of JAMES (1940, 3).

SCUDI, RATISH and BULLOWA (1939), and JAMES (1940, 2) showed that the urinary elimination of sulfapyridine runs parallel to the increased excretion of glucuronic acid. According to SCUDI and ROBINSON (1941), this increase was most distinctly noticed after the 5th day of the experiment when sulfapyridine was fed to the rats. Sulfathiazole produced this effect only in a lesser degree, while sulfanilamide was inactive, and so was *p*-hydroxybenzenesulfonic acid. THORPE and WILLIAMS (1940), however, noted a definite increase in the excretion of glucuronic acid by feeding sulfathiazole to rabbits. According to MARTIN and RENNEBAUM (1943), the conjugation of the sulfonamides with glucuronic acid is not so sharply limited, in that, for instance, even sulfanilamide increases the excretion of glucuronic acid if fed in large quantities. SCUDI and ROBINSON conclude that up till 40 % of the so-called "free sulfapyridine" may really be excreted with the urine in the form of glucuronate. That the site of the conjugation of sulfapyridine is in the liver was proved by damaging the liver tissue with phosphorus when the excretion of glucuronic acid was stopped completely. The same fact was revealed in the *in vitro*-experiments of LIPSCHITZ *et al.* (1939). These authors report that hydrocyanic acid and hypnotics exert a similar effect. Adrenalin was likewise found to decrease the ability of the liver tissue to form glucuronates while insulin increased their formation. Also kidney tissue is to some extent capable of this conjugation.

After an oral dose of the sulfonamide, SCUDI (1940) isolated from the urine of the dog the glucuronate of sulfapyridine, and SAMMON, SHELSWELL and WILLIAMS (1941) and WILLIAMS (1943), that of sulfanilamide. WEBER, LALICH and MAJOR (1943) have shown that in dogs, part of the sulfapyridine is excreted as a glucuronate attached to a hydroxyl group on the pyridine ring.

The formation of glucuronates reaches a maximum first on the 5th day of the experiment. Since it is known that acetylation reaches a maximum already on the first day, we may conclude that acetylation is a considerably more rapid reaction than the formation of the glucuronic acid derivatives. Convincing proof of the competition between acetylation and glucuronate formation was supplied by MARTIN, RENNEBAUM and THOMPSON (1941), and by MARTIN and RENNEBAUM (1943) who showed that addition of glucuronic acid to the diet decreases acetylation.

That there are still many obscure problems in the sulfonamide metabolism is clearly seen from these partly contradictory reports, and especially from the work of ALEXANDER (1943) with rabbits, sheep and mice. He determined the concentration in the blood and found it to be lower than calculated on the basis of a uniform distribution in the tissues. The higher values obtained for the concentration in the liver and kidney tissues could not account for this deficit. He therefore assumed that at least 30 % of the sulfanilamide is present in the tissues in some form as

yet unknown. In perfect agreement with this conclusion is SIMESSEN's (1941) report that during the first day up to 50 % of the sulfanilamide may be destroyed in mice, and somewhat less of the sulfathiazole. In 3 days, almost 50 % of sulfapyridine disappears, and up to 70 % of uliron.

In addition to the above conversions it should be remembered that some sulfonamides with large molecules are broken down into smaller components in the organism, mostly into sulfanilamide and a free side ring. TRÉFEOUËL *et al.* (1935) showed that the physiologically active part of the prontosil molecule is the sulfanilamide. ENGEL (1938) showed by *in vitro*-experiments that liver or kidney pulp or blood effect a cleavage of the prontosil molecule, liberating sulfanilamide. According to BRATTON *et al.* (1939), *p*-hydroxylaminobenzenesulfonamide is rapidly converted to sulfanilamide. BERNHEIM (1941) reports that when neo-prontosil is destroyed by liver pulp *in vitro*, the destruction starts with a rupture of the azo bond. FLYNN and KOHL (1941) found that in rats, *p*-nitrobenzenesulfonamide-pyridin is broken down *in vivo*, probably by the intestinal bacteria, to sulfapyridine, and *p*-nitrobenzenesulfonamide to sulfanilamide, both *in vivo* and, by liver pulp, also *in vitro*. SVARTZ, KALLNER and HELANDER (1945) have shown that salazopyrine undergoes cleavage in the organism, producing sulfapyridine. JAMES (1940, 3) could not establish a cleavage of sulfapyridine into simpler components, whereas KARCZANG and BEREND (1943) showed that the pyridine content of the expired air increases after intake of sulfapyridine. This they assume to be due to a rupture of the side ring of the sulfapyridine resulting in the liberation of pyridine. POTH and KNOTTS (1941) showed that succinyl sulfathiazole undergoes a partial cleavage to sulfathiazole in the intestinal tract.

Excretion of sulfonamides.

The sulfonamides are excreted chiefly with the *urine*, partly free and partly in the acetylated form. According to FRISK (1941, 2), the clearance values for sulfanilamide, sulfapyridine, sulfathiazole and sulfamethyl thiazole in man are $1/3 - 1/2$, $1/5$, $1/2$ and $1/6$, respectively, of the creatinine clearance. With all these compounds the clearance of the acetylated form was higher, due to the lesser resorption back of the acetylated form from the tubules. The clearance values were independent of the concentration in the blood but proportional to the quantity of the *urine*. When a single oral dose of 5 g is given, the urinary excretion within 24 hrs accounts for 40—50 % of the acetylated sulfanilamide, 10—30 % of acetylated sulfaguanidine, 50—70 % of acetylated sulfathiazole and 20—60 % of acetylated sulfapyridine, the corresponding values for total excretion being 90—97, 15—50, 80—90 and 70—80 %.

Excretion with *faeces* occurs particularly with sulfonamides, which are only sparingly soluble or poorly resorbed. A very small proportion of the sulfonamide in the *faeces* has been really absorbed in the alimentary

canal and then excreted with the bile. Hence it follows, for instance, that only traces of sulfaguanidine are found in the faeces after a subcutaneous injection of the drug. According to HAWKING (1942), the elimination of sulfaguanidine in the faeces of man after an oral dose is greater than that of the other sulfonamides. In cats, all the drugs are excreted at about the same rate while in the faeces sulfathiazole is excreted best in mice. MARQUARDT (1938) and REIMERS (1939) report that also uliron is excreted in large quantities in the faeces. — Prolonged use of the drugs increases the faecal excretion with all compounds.

Other possible ways of excretion. Sulfonamides are secreted in the other excretions and secretions in concentrations similar to those in the blood. The dog experiments of COOKE, DAVENPORT and GOODMAN (1941) showed that even after parenteral injections of sulfathiazole, sulfadiazine and acetyl sulfanilamide, the drugs are secreted in the stomach in concentrations almost equalling those in the blood, and that values exceeding those in the blood may be obtained with sulfanilamide or sulfapyridine. This is probably the explanation of the vomiting which occurs in patients receiving the latter preparations. The secretion is obviously a process of physical diffusion and is independent of the acidity of the gastric juice. However, according to HÜLLSTRUNG (1938), the acidity of the gastric juice falls when sulfonamides are being used over longer periods.

According to TURKELL and WILHELM (1941), the concentration of sulfathiazole in tears is less than 1 mg-% when clinical doses are given. The concentration in tears does not follow that in the blood, and is not associated with the allergic conjunctivitis.

When clinical doses are given, the concentration of excreted sulfanilamide in sweat is 2/3 of that in the blood (CORNBLUT, 1940). The concentration of sulfanilamide in the saliva is 50—100 %, and in *pancreas* and *prothata fluids* about 75 % of the blood concentration (HUG, 1940). The drug is also excreted, mainly by way of the prothata, into the spermatic secretion, where it can be still found 2—3 days after the conclusion of the therapy (BONADONNA and COMI, 1943). In the *urethral secretion* sulfonamides appear in concentrations similar to those of the blood (BIERING—SÖDERSEN, 1943). Small amounts of sulfonamides appear also in the *menstrual blood* and in the *cervix-secretion*.

In view of the central position of the liver in the sulfonamide metabolism it is quite natural that special attention has been paid to the secretion of the sulfonamides in the bile. Besides, secretion in the bile might have also clinical importance, for instance, in cases of cholecystitis. In general, the concentration of all sulfonamides — with the exception of sulfapyridine — in the bile is lower than that in the blood, particularly as regards the acetylated form. The low concentration of the acetylated form is rather remarkable in view of the fact that acetylation is known to take place in the liver.

SPINK, BERGH and JERMESTA (1941) gave patients with choledochus-

fistula clinical doses of sulfanilamide and sulfapyridine *per os* and found that the concentration of sulfanilamide in the bile was considerably less than in the blood, whereas in 2 patients out of 3, the bile contained more sulfapyridine than did the blood. There were only traces of the acetylated forms in the bile. Even when acetyl sulfanilamide was given orally, its concentration in the bile remained below that in the blood. Approximately identical results were obtained by HUBBARD and BUTSCH (1941) who used also sulfathiazole. The concentration of the latter drug in the bile was only 1/5—1/20 of the blood concentration. In another paper (1941) they found the ratio of the concentrations of sulfaguanidine in the bile and in the blood to be 1:2. SIEDE (1943) studied the excretion of albusid, globusid, pyrimal, sulfathiazole and sulfapyridine in the bile, and found — in agreement with GOUGH (1943) — that the concentration of sulfapyridine in the bile exceeded that in the blood. The said authors therefore conclude that in cases of cholecystitis sulfapyridine could be expected to give the best results. The animal experiments of AGREN and TAYLOR (1940) confirm the secretion of sulfapyridine.

BARBER *et al.* (1943) prepared a compound of sulfanilamide with bile acid, which is mostly excreted in the bile but whose clinical use is restricted by the fact that its bacteriostatic effect is limited to streptococci. It is quite inactive, for instance, against *E. coli*.

Excretion in the milk of lactating women. The concentration of the sulfonamides in the milk of lactating women generally remains below that in the blood, irrespective of the compound used or the mode of dosage (STEVARD and PRATT, 1938; HAC, ADAIRE and HESSELTINE, 1939). The ratio of free to conjugated form is similar to that in the blood. With therapeutic doses, CHOSSON and ANDRAC (1943) noted a secretion of 5—6 mg per day. In general, the concentration varied between 1 and 13 mg-%. Hence it is safe to conclude that the small amount of sulfonamide appearing in the milk of the mother can have no effect, toxic or therapeutic, on the infant, nor can it influence its increase of weight (PINTS, 1938; RIEBEN and DRUEY, 1942). On the other hand, it may have an allergenic effect as evidenced by a case reported by ELGENMARK (1945). He found allergic skin rash in the lip of an infant who had been treated for common cold with a sulfonamide and whose mother had shortly before been given a course of the same compound. In this case the sulfonamide in the mother's milk had acted as the allergenic factor while the oral dose produced the trigger effect.

Part II

Own investigations.

Chapter 1. Effect of the sulfonamides on the microbial synthesis of the B complex vitamins, and particularly of vitamin B₁ in the intestines of the rat.

Outlining of the problem.

Various authors have assumed that the sulfonamides produce a deficiency of thiamine (vitamin B₁) in the organism and that this is — at least in part — the cause of the neurotoxic complications observed in connection with sulfatherapy. *To explain how the B₁-deficiency may arise, MacLEAN and BENESCH (1944) were the first to assume that sulfonamides inhibit the synthesis of vitamin B₁ in the intestinal tract.* It was therefore of interest to study the synthesis of vitamin B₁ in the intestines of the rat, and the effect on this synthesis of various sulfonamides (sulfanilamide, sulfapyridine, sulfathiazole, sulfametine, salazopyrine, sulfadigesine, sulfadimine, sulfaguanidine, uliron, neo-uliron, albusid, marfanil-prontalbine and marfanil.pur.) and of certain related compounds (p-aminobenzoic and sulfanilic acids).

Earlier work.

Synthesis of vitamins in the intestinal tract and the conception of "refection".

The discovery of the intestinal vitamin synthesis can be ascribed to FRIDERICIA, FREUDENTHAL, GUDJONSSON, JOHANSEN and

SCHOUBYE (1927) since they were the first to establish a synthesis of thiamine in the rat intestine.

Starting from the ground laid by FRIDERICIA, recent research on vitamins and sulfonamides has revealed that in the intestinal tract of several mammals (man, dog, cow, sheep, monkey, rat, mouse), micro-organisms synthesize, in addition to thiamine, also a number of other vitamins, particularly those of the B complex: nicotinic, pantothenic and folic acids, pyridoxine, riboflavin and biotin, as well as vitamin K.

It has been known ever since the earliest phases of vitamin research that symptoms of thiamine deficiency cannot be produced in rats if coprophagia is not excluded. It was apparent, therefore, that the faeces of rats contains some factor which prevented the development of thiamine deficiency. The possibility that this factor was thiamine, stored in the tissues of the animal and removed with the excreta, could be excluded by prolonging the experiment sufficiently for all stored thiamine to be either utilised or excreted. The only remaining possibility, therefore, was a synthesis of B₁ in the organism, particularly in the intestines. In fact, FRIDERICIA *et al.* showed that the crude starch given to the rats, contained micro-organisms capable of synthesizing thiamine. They could not identify this synthesizing micro-organism, but it could be transferred from animal to animal if the faeces of one animal was fed to another. This phenomenon is called "refection", according to FRIDERICIA. Investigations on refection have been carried out also by ROSCOE (1927).

The vitamin B balance.

Before entering into the question of the intestinal synthesis of vitamin B₁, it is necessary to discuss certain factors which influence the balance and excretion of this vitamin. Vitamins of the B complex are eliminated both with the urine and with the faeces. The extent of elimination depends on the amount administered with the food, the quantity stored in the body, utilisation of the vitamin, and on the intestinal synthesis. The view is generally held that all the above factors, with the exception of intestinal synthesis, influence the elimination with the urine, whereas on a normal diet, elimination with the faeces depends chiefly on synthesis. Thus, WILDEMAN's (1941) rat experiments showed that the elimination of thiamine in the faeces was practically independent of the oral administration. The urinary elimination, on the other hand, was closely correlated with the quantities fed *per os*. WILDEMAN therefore concluded that the thiamine balance can be estimated accurately enough by observing the excretion of the vitamin in the urine. In his opinion, the amount eliminated with the faeces is a result of the bacterial synthesis. LEONG (1937) likewise states that in rats the elimination of B₁ in the faeces is independent of the oral administration provided this does not exceed 30 I. U. per day. NAJJAR and HOLT (1943) have shown that in man the elimination of thiamine in the urine is proportional to the amount

received *per os* whereas the elimination in the faeces is independent of it.

According to SCHRÖDER and LIEBICH (1939), and LINNEWEH and MÜLLER (1940), the resorption of thiamine and other B complex vitamins occurs only in the upper part of the alimentary canal. There are, however, certain facts which speak for a resorption in the coecum, and in the large intestine in general. Thus, NAJJAR and HOLT found that the elimination of thiamine in human urine increased manifold when the subject was given 50 mg of thiamine *per rectum*. The view that resorption occurs in the lower parts of the alimentary canal, is supported also *e.g.* by the results of NAJJAR and HOLT and of ELLINGER *et al.* (1944, 1945, 1, 2), who studied the intestinal synthesis in B-deficient persons.

Effect of diet on the intestinal synthesis of the B complex.

The observation that vitamins are synthesized by micro-organisms in the alimentary tract immediately led to a study of certain external factors like diet *etc.*, which could be expected to influence these bacterial functions.

GUERRANT *et al.* (1935) found *i.a.* that if the carbohydrate constituent of the diet was dextrinized corn starch, which arrives in the large intestine partially unchanged, the production of the B complex vitamins in the intestinal tract was higher than when the rat was fed easily-digestible carbohydrates. Also MITCHELL *et al.* (1942) have shown that different carbohydrates promote to a different extent the synthesis of the B complex vitamins.

WHIPPLE and CHURCH (1935) found that a fat-free diet caused an earlier appearance of B-(B₁)-deficiency symptoms in rats than did fat-containing diets. Dry faeces, which was extracted with ether, gave a better protection against the deficiency symptoms than the extract, so the fat itself was inactive.

Use of sulfonamides in the research on the intestinal synthesis of the B complex.

Experimental difficulties caused the research on the intestinal vitamin synthesis to remain at this stage until the use of sulfonamides as paralyzers of the microbial action opened up new ways of approach.

Intestinal synthesis of vitamin B₁. NAJJAR and HOLT (1943) studied the synthesis of thiamine in the human intestinal tract in order to settle the disagreement in reports of the minimum thiamine requirements of man, which had appeared in literature (*e.g.* KEYS and HEUSCHEL, 1942; KEYS, 1943). The subjects were kept on a B₁-free diet for several months. It was found, however, that only a few persons developed deficiency symptoms while the others remained fully fit.

During such a long experimental period the stores of the body were certainly exhausted, which was best illustrated by the complete cessation of any excretion of thiamine in the urine. Consequently, thiamine must be synthesized somewhere and in this respect examination of the faeces gave the solution. It was found that the faeces of persons with deficiency symptoms contained practically no thiamine while considerable quantities were found in the faeces of symptom-free persons. Succinyl sulfathiazole treatment, applied in one case, diminished the elimination of thiamine in the faeces to almost nil. This proved beyond doubt a synthesis by the intestinal flora, which synthesis was inhibited by the bacteriostatic action of the sulfonamide.

The authors concluded that when the intake of thiamine is very slight or nil, the intestinal synthesis plays a significant rôle also in man as a protection against the deficiency symptoms. The synthesizing ability, however, varies with different individuals, and this difference is largely responsible for the divergent results obtained in investigations on the minimum requirements of thiamine. It is possible, however, that some thiamine must be received from outside, at least from time to time, to stimulate the intestinal bacteria. That such a stimulation of the intestinal synthesis really occurs was shown by WEGNER, BOOTH, ELVEHJEM and HART (1940). ELVEHJEM (1941) has likewise shown that administration of pantothenic acid increases the synthesis of the B complex vitamins which, according to MARTIN (1943), depends also on *p*-aminobenzoic acid.

LAURENT and SINCLAIR (1938) found that in an acylic stomach thiamine is destroyed so that only small amounts are absorbed in the intestines. According to STOCKHOLM, ALTHAUSEN and BORSON (1941), the destruction of thiamine by acylic stomach is further increased by the partial secretion back to the stomach of the thiamine absorbed in the intestines. HÜLLSTRUNG (1938) again showed that in some cases uliron diminishes the secretion of hydrochloric acid into the stomach and that, especially in such cases, there is a decrease in the urinary elimination of thiamine.

Abundant vitamin synthesis has been shown to take place in the alimentary canal of ruminants, particularly in the rumen. McELROY and GOSS (1939, 1940, 1, 2, 3, 1941) studied the synthesis of the B complex vitamins in sheep and calves. Using a B complex-free diet they found that there are produced in the rumen, in addition to other B complex vitamins also riboflavin, pyridoxine and pantothenic acid. WEGNER *et al.* (1940) studied the same synthesis in the calf and found that there are produced in the rumen: thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and biotin. As the synthesis of thiamine seemed slight compared to that of, *e.g.* nicotinic acid — being only 5% of the latter — it was near at hand to assume that part of the thiamine is also destroyed in the rumen. In order to settle this point, a known amount of thiamine was included in the diet. It was established that there is no destruction of thiamine. At the same time it was observed that the added thiamine

stimulated the bacterial activity, as evidenced by an increased production of the other vitamins. Pantothenic acid has been found to act similarly.

The extent of thiamine synthesis in ruminants surpasses by far that *e.g.* in the alimentary tract of man. This is shown, for instance, by the fact that the thiamine content of the milk of lactating women runs parallel to the thiamine content of the diet, while that of cow's milk is largely independent of the latter. Suckling calves therefore thrive well even when their mother's diet is devoid of the B complex vitamin (McELROY and GOSS, 1940, 1). — KON (1927) has found that thiamine synthesis occurs also in the intestinal tract of the pigeon.

Intestinal synthesis of nicotinic acid. *) SHUORIE and SWAMINATHAN (1940) found that the rat is capable of synthesizing nicotinic acid but they did not locate the site of this synthesis. DANN and KOHN (1940), and DANN (1941), determined the amount of nicotinic acid in the tissues of rats kept on a low-nicotinic acid diet. In order to designate the site of the synthesis DANN added 1% sulfaguanidine to the diet to inhibit possible synthesis in the intestines. The increase of body weight of the so-treated rats was stopped and an administration of nicotinic acid *per os* did not cause an increase of weight, as was the case with controls. DANN therefore concludes — in part erroneously — that the change in the increase of body weight in the sulfaguanidine group was not ascribable to an inhibition of the intestinal synthesis, but merely to toxic effects of sulfaguanidine on the rat tissues. He pays no attention to the fact that sulfaguanidine may also have inhibited the intestinal synthesis of certain other factors which influence the increase of weight in young rats (NIELSEN and ELVEHJEM, 1942, *etc.*). The diet he used did not contain these factors. DANN thus concludes from his experiments that the synthesis of nicotinic acid occurs exclusively outside the intestinal canal. In this opinion, the nicotinic acid synthesis in the tissues is so extensive that he — in contrast to v. EULER *et al.* (1939) — does not regard nicotinic acid as a vitamin for the rat. According to DANN, the rat is thus self-supporting as regards this factor.

SCHAEFER, McKIBBIN and ELVEHJEM (1942, 2) have found *i.a.* that milk diet increases the intestinal synthesis of nicotinic acid in the dog; the effect is not due to lactose. ISBELL (1942) has shown that *p*-aminobenzoic acid stimulates the ability of certain bacteria to produce nicotinic acid. The curative effect of oral doses of nicotinic acid on dogs suffering from nicotinic acid deficiency was inhibited when sulfapyridine was simultaneously administered. However, when fresh liver extract was also given, the inhibiting effect of sulfapyridine did not appear. In SCHAEFER's opinion, sulfapyridine either inhibits the formation of

*) SIMOLA showed already 1932 that vitamin B-free diet does not lower the concentration of nicotinic acid in the rat tissue, while it does lower the cocarboxylase content. — He was thus also the first to show that there is an interrelation between the vitamin B metabolism of the organism and cocarboxylase.

cozymase or its utilisation after it has been formed from nicotinic acid. BICKNELL and PRESCOTT (1946) likewise assume that there exists between sulfapyridine and nicotinic acid a similar antagonistic effect as between sulfanilamide and p-aminobenzoic acid. This view is supported not only by bacteriological observations but also by purely clinical experience [see, for instance, COTTINI (1940)] which shows that nicotinic acid also prevents several unfavourable secondary effects of sulfapyridine.

ELLINGER *et al.* (1944, 1945, 1, 2) showed that also in the intestinal canal of man, micro-organisms synthesize nicotinic acid. The experiments were made using succinyl sulfathiazole and sulfathiazole as inhibitors of the bacterial action. It was found that succinyl sulfathiazole caused a decrease of up to 60 % in the excretion of nicotinic acid in the urine whereas no definite effect was noted with sulfathiazole. The effect of succinyl sulfathiazole was ascribed, in the first place, to its poor solubility. On the basis of the relatively high excretion values obtained, the authors assume that intestinal synthesis of nicotinic acid plays a rôle also in the prevention of deficiency symptoms when the diet is low in nicotinic acid.

Intestinal synthesis of other B complex vitamins. It appeared from the discussion of the thiamine synthesis in the rumen of ruminants, that in addition to thiamine and nicotinic acid, also other B complex vitamins have received their share of attention in the elucidation of the problem. This applies, in the first place, to riboflavin, pyridoxine, pantothenic acid, biotin and folic acid, most of which have been recognised as growth factors of the immature animal (DAFT, ASBURN, SEBRELL, 1942; SCHWARZ, 1942). According to MITCHELL *et al.* (1942) the synthesis of the B complex vitamins in the rat takes place in the following quantitative order: biotin, pyridoxine, folic acid, pantothenic acid, thiamine and riboflavin.

In their study of the riboflavin balance in man, NAJJAR, JOHNS and MEDAIRY (1944) found the elimination in the urine and excreta to exceed the intake of riboflavin with the food. They therefore assume that a synthesis takes place in the organism, probably in the intestine. However, administration of succinyl sulfathiazole did not stop the synthesis.

Pantothenic acid has been recognised as growth factor already for several years. SCHAEFER, McKIBBIN and ELVEHJEM (1942, 1) have shown that pantothenic acid deficiency occurs e.g. in intestinal disorders. WEST *et al.* (1943) could produce such deficiency in the rat by feeding sulfapyridine. The deficiency symptoms disappeared when pantothenic acid was given. WRIGHT and WELCH (1943) likewise noticed symptoms of pantothenic acid deficiency by feeding sulfathiazole. Curiously enough, these symptoms could not be made to disappear with pantothenic acid unless biotin and folic acid were simultaneously given. The said substances have therefore been considered essential for the synthesis of pantothenic acid. According to GORDON (1942) the synthesis of pantothenic acid in man is comparatively slight.

BLACK, McKIBBIN and ELVEHJEM (1941) were the first to pay

attention to the fact that in young rats, kept on a biotin and folic acid-free diet, administration of sulfaguanidine either retarded or completely checked the increase of body weight. The control group, receiving no sulfonamide showed a normal increase of weight. Feeding of p-aminobenzoic acid or liver prevented this inhibiting effect. The reason why sulfaguanidine checks the increase of body weight is that the intestinal synthesis of biotin and folic acid is inhibited. Liver extract, containing these very factors, neutralised the effect of sulfaguanidine, — and so did p-aminobenzoic acid.

NIELSEN and ELVEHJEM (1942) showed that the cessation of weight increase, caused by the above diet and succinyl sulfathiazole, could be corrected by giving biotin and folic acid. DAFT, ASBURN and SEBRELL (1942) found that rats which, in addition to purified diet, received succinyl sulfathiazole and sulfaguanidine, in the absence of biotin developed granulocytopenia, leucopenia, hypercellularity of bone marrow, hyaline sclerosis and calcification of blood vessels, and also skin rash which responded favourably to treatment with (crystalline) biotin. Necrosis of the heart muscle was also noted, as well as hemorrhages in different organs and under the skin, and changes in the liver. WELCH and WRIGHT (1943), and BLACK *et al.* (1942) have likewise found that certain sulfonamides cause symptoms characteristic of biotin and folic acid deficiency. It may be mentioned here that already in 1940 MacHELLA and HIGGINS observed that sulfonamides prevent or retard the normal increase of body weight in rats, although they could not associate their results with the effect of the sulfonamides on the bacterial flora in the intestines of the rat.

Micro-organisms, responsible for the intestinal synthesis of the B complex vitamins, and the site of the synthesis. An interesting feature of the problem is to ascertain which micro-organisms partake in the intestinal synthesis of the B complex vitamins and in which part of the alimentary tract this process occurs. FRIDERICIA and his collaborators started from the fact that the micro-organism, which is responsible for the intestinal synthesis of thiamine, was carried into the intestines of the rat together with crude starch. GUERRANT *et al.* (1935) showed that yeasts isolated from rat coecum were capable of synthesizing those factors which prevent the appearance of symptoms of B complex deficiency. Further evidence was supplied by HEDSTRÖM and HÖGLUND (1944), whose experiments have gained also practical significance. They fed well-identified strains of yeast to suckling calves and showed that these strains produce in the alimentary tract of the calf, probably in the rumen, essential growth factors which had been earlier studied by McELROY and GOSS (1939, 1940, 1, 2, 3, 1941). WIELAND and MÖLLER (1941) paid attention to the ability of yeasts to synthesize pantothenic acid *in vitro*. There are scattered notes in literature that *Bacillus mesentericus* in the intestine of herbivorous animals, *Escherichia coli*, occurring abundantly e.g. in the intestines of man, and *Lactobacillus bifidus* in the suckling

intestine are capable of synthesizing at least thiamine (WILLIAMS and SPIES, 1938). It was not possible, however, to obtain a reliable microbe analysis until the sulfonamides were taken into use as paralyzers of the microbial action.

GANT *et al.* (1943) studied the effects of sulfaguanidine and succinyl sulfathiazole on the counts of *E. coli* and *enterococci* in the rat. It appeared that the content of bacteria, particularly of *E. coli*, in the stomach was abundant only when the rat had had access to its own excreta. If this was effectively prevented, the stomach was practically bacteria-free. The bacterial content of the duodenum was constant, provided that there were not very large amounts of *coli* in the stomach. The bulk of bacteria in the intestinal tract was found in the colon and the coecum. In both, it was very similar and showed similar changes during the experiment. This is in agreement with the work of GUERRANT *et al.* (1935) who showed that the most important site of the B complex synthesis is in the coecum. GUERRANT arrived at this conclusion by comparing the abilities of normal and coecetomized rats to synthesize vitamins of the B complex in the intestinal tract. MITCHELL *et al.* (1942) likewise stress the importance of the synthesis in the coecum.

GANT's *et al.* work further showed that, from the third day on, the count of *E. coli* fell rapidly in the intestines and excreta, remaining low for about 3 weeks, whereupon it again began to rise, reaching soon its earlier level. However, the total number of micro-organisms remained constant, in spite of the decrease in the numbers of *coli*. This was explained by a simultaneous increase in *enterococci*. Similar phenomena had been observed by LIGHT *et al.* (1942) in rats fed with sulfaguanidine, MARSHALL *et al.* (1940) in man, WELCH *et al.* (1942) in the monkey and by POTH *et al.* (1942) in the dog.

Merely the facts that the numbers of certain bacteria decrease or that sulfonamides cause variations in the proportions of different bacterial species, would have said very little of the intestinal synthesis by bacteria, had not the changes in the host organism been also observed. Simultaneously with the decrease of the *coli* group in rats on purified diet, general symptoms of B complex deficiency could also be noted, particularly as regards the increase of body weight. This was best seen in those groups which showed the most marked decrease of the *coli* group.

The rise of the *coli* group to its earlier level is probably due to a gradually developing chemoresistance. However, the bacterial strain thus changed is different also in another respect: it has lost its ability to synthesize vitamins. This is best illustrated by the fact that there was no adequate rise of the body weight parallel to the increase of the *coli* group.

Hence it can be concluded that *E. coli*, which in rats is found mostly in the coecum, takes part in the synthesis of the B vitamin complex, whereas the *enterococci* do not.

THOMPSON (1942) found that *Aerobacter aerogenes*, under similar conditions, synthesizes biotin and folic acid.

MILLER's (1944) work with succinyl sulfathiazole and phthalyl sulfathiazole forms a complement to GANTS's *et al.* investigations. Of the two compounds, phthalyl sulfathiazole proved to inhibit more effectively the activity of the intestinal bacteria. In addition to aerobic bacteria, also anaerobic species were studied and found to undergo similar variations in numbers as *E. coli*. ELLINGER and BENESCH (1945) made corresponding experiments with man and reported great individual variations in the counts of bacteria before and after the sulfonamide treatment, although the general trend was similar to that in animals.

Experimental.

It is natural to expect that the size of the rat would influence the faecal and urinary output of thiamine — already because the amount of the excreta depends on the size of the animal. It was shown above that also diet plays a rôle in this respect.

For these reasons, the present research on the intestinal synthesis of thiamine in the rat was divided into two parts:

A. *Preliminary investigations on the elimination of thiamine in different weight groups, and on the effect of diet on this elimination.*

B. *Investigations on the effect of the sulfonamides and related compounds on the synthesis of vitamin B₁ in the intestines of the rat.*

A. In the preliminary investigations the rats were divided into weight groups of 220 g (± 10 g) and 110 g (± 10 g). Attention was paid to changes in the body weight and to the elimination of vitamin B₁ in the urine and stools on laboratory standard diet, vitamin B₁ control diet, B₁-deficient diet and completely B-free (yeast-free) diet.

Experimental procedure. The controls consisted of 30 male rats, of which at the start of the experiment one-half belonged to the 220 g (± 10 g) group and the other half to the 110 g (± 10 g) group. Both groups were again divided into 3 groups of 5 rats each. The first of these groups was given vitamin B₁ control diet, the second B₁-deficient diet and the third B₁-free diet. In each group the excretion of vitamin B₁ was first followed during a week's time on standard diet, before the experimental diet was begun.

The vitamin B₁ control diet consisted of 225 g mixed flour, 36 g fat, 40 g dry baker's yeast, 2 1/2 gtt "Vigantol" and "Vogan" (Bayer), and 200 ml water. The B₁ deficient group was given a corresponding quantity of autoclaved baker's yeast, while no yeast was given in the B-free diet. The flour mixture initially consisted of 1000 g rice-starch, 400 g alcohol-

extracted casein and 100 g of the following salt mixture: 1600 g KH_2PO_4 , 1200 g $\text{Ca}(\text{H}_2\text{PO}_4)_2$, 400 g NaCl , 400 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 400 g calcium lactate, 40 g FeCl_3 and 1.2 g KJ . The food mixture and water were given *ad libitum*.

Owing to exceptional circumstances, however, the rice-starch had to be replaced by potato flour. Before the potato flour was taken into use, its B_1 -content was determined. It appeared that the average B_1 -content of unautoclaved potato flour was 60 γ -%, and of autoclaved flour 15–20 γ -%, while rice-starch contained, on the average 10 γ -% of B_1 . In this respect, autoclaved potato flour thus proved a suitable substitute for rice-starch. (The average B_1 -content of wheat flour was 5 to 10 times higher than that of potato flour. *)

The experimental period was 6 weeks. The rats were weighed weekly; from these weighings the mean weights in each series were calculated. The stools and urine were collected daily and analysed every second day. The urine was collected with the aid of a funnel, in erlenmeyer flasks, while the stools were collected in a net placed over the funnel. Estimation of vitamin B_1 was carried out mainly according to RITSERT's (1938, 1, 1940) thiochrome method, with slight modifications. To 1 ml of the urine were added 19 ml water and 0.3 ml 25 % hydrochloric acid. The mixture was heated for 5 to 6 mins on a 50° C water bath with occasional shaking whereupon it was filtered hot through moist filter-paper. 10 ml of the clear filtrate was washed twice with isobutyl alcohol in a separating funnel. 20 ml of 15 % NaOH were now added with shaking, and then 1 % potassium ferricyanide until a definite yellowish colour was obtained. The thiochrome formed was extracted into 20 ml isobutyl alcohol. The aqueous layer was separated and the isobutyl alcohol layer dried with anhydrous Na_2SO_4 . Thiochrome was then determined in an aliquot of 10 ml isobutyl alcohol — corresponding to 0.5 ml urine — by comparing its fluorescence against that of a standard thiochrome solution in isobutyl alcohol.

The standard thiochrome solution was prepared by oxidizing 2 mg thiamine (Betaxin amp.) to thiochrome and diluting it, prior to use, with isobutyl alcohol in the ratio of 1 to 50 so that 1 ml of this solution corresponded to 1 γ of thiochrome. In the estimation of the fluorescence, standard thiochrome solution was buretted into 10 ml of pure isobutyl alcohol until the fluorescence equalled that of the solution to be examined.

The stools were ground with quartz sand in a mortar into a homogeneous mass, which was then extracted with a suitable quantity of water (RITSERT, 1938, 2). The solid residue was filtered off and thiamine was determined in the filtrate in the same manner as in the urine.

Since it was not known, whether the fluorescence of the sulfonamides interferes with the estimation of thiochrome, a series of control experiments was made to determine the possible fluorescence of known sulfon-

*) Autoclaving lowered the vitamin B_1 content of baker's yeast from 300–360 γ -% to 40–50 γ -%.

amide solutions. For this purpose, 10, 50, 100, 150 and 200 mg-% solutions of sulfapyridine, sulfathiazole and sulfanilamide were prepared in rat urine and the thiamine content of the solutions was determined. No differences could be noted in the fluorescence values. Consequently, the own fluorescence of the sulfonamides does not interfere in any way with the estimation of thiamine.

The results of the control experiments are given in the corresponding tables. Table 1 shows the results in the weight group of 220 g (± 10 g), and Table 2 in the weight group of 110 g (± 10 g), on vitamin B₁ control diet, B₁-deficient diet and on altogether yeast-free diet.

TABLE 1, illustrating the changes in body weight, and the urinary and faecal elimination of vitamin B₁ per rat and day on three different diets.
5 rats in each series. Weight group 220 g (± 10 g).

Vitamin B ₁ control diet			Vitamin B ₁ deficient diet			Yeast-free diet		
Elimination of vitamin B ₁ per rat, γ		Weight, g 221	Elimination of vitamin B ₁ per rat, γ		Weight, g 223	Elimination of vitamin B ₁ per rat, γ		Weight, g 227
urine	faeces		urine	faeces		urine	faeces	
3.3	2.5	228	3.0	3.3	231	2.7	4.1	233
2.9	2.7		3.3	2.6		2.9	3.0	
3.0	2.3		3.2	2.5		3.4	2.7	
3.1	2.1		3.2	3.1		2.5	3.1	
Transfer from standard to experimental diet								
2.1	2.3	232	2.6	2.2	231	2.1	2.9	224
1.7	2.0		1.4	2.5		2.4	3.1	
1.4	2.6		1.3	2.5		1.8	2.4	
1.8	2.1		1.5	2.8		1.1	2.1	
1.7	2.3	236	1.1	2.8	233	1.7	2.5	215
1.4	2.1		1.1	2.3		1.2	2.1	
1.4	2.5		1.2	3.1		1.0	1.8	
1.5	2.7		1.1	2.1		0.6	1.7	
1.8	2.4	248	1.3	2.4	240	0.7	1.5	213
1.6	2.3		1.2	2.6		0.3	2.1	
1.6	2.8		1.3	2.5		0.6	2.1	
1.3	2.7		1.0	2.1		0.5	1.7	
1.5	2.5	260	1.1	2.4	242	0.3	1.5	207
1.6	2.5		1.3	2.4		0.4	1.9	
1.6	2.2		1.2	2.7		0.3	1.8	
1.5	2.0		1.2	2.1		0.5	1.5	
1.6	2.2	270	1.0	2.1	247	0.3	1.7	207
1.4	2.0		1.2	2.2		0.3	1.7	

TABLE 2, illustrating the changes in body weight, and the urinary and faecal elimination of vitamin B₁ per rat and day on three different diets.
5 rats in each series. Weight group 110 g (\pm 10 g).

Vitamin B ₁ control diet			Vitamin B ₁ deficient diet			Yeast-free diet		
Elimination of vitamin B ₁ per rat, γ		Weight, g 110	Elimination of vitamin B ₁ per rat, γ		Weight, g 108	Elimination of vitamin B ₁ per rat, γ		Weight, g 117
urine	faeces		urine	faeces		urine	faeces	
2.2	1.3		1.4	0.8		2.1	1.1	
1.3	1.1		2.7	0.7		2.7	0.9	
1.9	0.7	133	2.1	1.5	125	2.0	1.3	137
Transfer from standard to experimental diet								
1.3	0.9		0.9	0.9		1.7	1.2	
1.0	1.1		1.4	0.9		1.3	1.3	
1.7	1.1	159	1.3	1.3	128	1.0	1.1	138
1.0	0.8		1.0	1.2		0.8	0.8	
0.9	1.3		0.8	1.3		0.7	0.9	
1.5	0.9		0.9	0.8		1.2	1.1	
1.1	0.8	193	1.2	0.9	155	0.9	0.3	144
1.3	1.1		0.7	0.9		0.5	0.9	
1.0	1.3		0.2	1.1		0.6	1.0	
1.1	0.8		0.6	0.7		0.3	0.3	
1.1	0.9	207	0.9	0.7	171	0.2	0.5	159
0.9	0.8		0.5	1.2		0.3	0.8	
0.9	0.9		0.3	1.1		0.1	0.4	
0.9	1.3		0.7	0.9		+	0.3	
0.9	1.1	214	0.3	1.3	188	+	0.5	171
1.3	0.9		0.5	1.1		+	0.4	
0.9	0.8		0.3	0.7		0.3	0.2	
1.1	1.3	228	0.4	0.9	191	0.2	0.7	183

The results obtained with rats which, at the beginning of the experiment, belonged to the 220 g (\pm 10 g) group, show that on standard diet the elimination of vitamin B₁ in the urine varied on both sides of 3 γ , and in the stools between 2 and 3 γ per rat per day, in all three groups. A transfer of the animals on vitamin B₁ control diet lowered the elimination in the urine to a level between 1.3 ja 1.8 γ per day. The values became fairly constant at the beginning of the second week from the transfer. A transfer into B₁-deficient diet lowered the elimination in the urine to values between 1 and 1.3 γ per rat and day, the values becoming practically constant at the end of the second week from the trans-

fer. In both groups the elimination of vitamin B₁ in the stools seemed to be independent of the change of diet. On yeast-free diet the elimination in the urine decreased more slowly, reaching a constant level — 0.3—0.5 γ per rat per day — only on the 3rd or 4th week from the transfer. In this group, also the elimination of thiamine in the stools was found to decrease. These values varied from 1.5 to 2 γ per rat per day, from the 3rd or 4th week on.

An examination of the weight curves shows that there was increase of weight on vitamin B₁ control diet and B₁-deficient diet, more in the former group, whereas on yeast-free diet the weight decreased during the experiment. *) The latter result is probably due to a deficiency in the diet of either protein or factors of the vitamin B complex.

The results in weight group 110 g (± 10 g) differ from these discussed above in the following respects: On standard diet the elimination of thiamine in the urine varied on both sides of 2 γ , and in the stools from 0.8 to 1.3 γ per rat and day. A transfer on vitamin B₁ control diet, or B₁-deficient diet caused a fall of the urinary elimination to a level between 0.9 and 1.3 γ , and from 0.2 γ to 0.7 γ per rat and day, while the elimination in the stools remained practically unchanged. A transfer on yeast-free diet caused also in this weight group the most marked decrease in the urinary elimination: the values attained constancy at a level which varied from 0 to 0.3 γ per rat and day. On this latter diet the elimination in the stools likewise fell, varying from the 3rd or 4th week onwards on both sides of 0.5 γ . There was an increase of weight in all groups, the increase being lowest on yeast-free diet.

Conclusions from the control experiments. The relatively high elimination of thiamine in the urine on standard diet shows that, even under the present exceptional conditions, the food given to the rats contained a sufficient amount of vitamin B₁. The decrease of the urinary elimination on vitamin B₁ control diet again shows that this contains less thiamine than the standard diet. On the other hand, transfer of the animals from standard diet into con-

*) It is quite certain that autoclaving destroys also other vitamins of the B complex besides thiamine, for instance growth factors (KRUSIUS and SIMOLA, 1937; SIMOLA, 1939, 1944)

trol or B₁-deficient diets had no effect, in either of the weight groups, on the elimination of thiamine in the stools. It is apparent, therefore, that at least on these diets, elimination of thiamine in the stools is independent of the quantities of thiamine contained in the food (cf. WILDEMAN, 1941). In fact, there is full reason to regard the thiamine contained in the stools as a result of the intestinal synthesis.

The differences noted in the urinary elimination on B₁-deficient and yeast-free diets, respectively, are evidently due to the fact that the former diet contained some thiamine. The thiamine which is eliminated in the urine on yeast-free diet is probably derived from the thiamine stores of the body. NAJJAR and HOLT's (1943) experiments with man have shown that from such stores thiamine is excreted into the urine, on a thiamine-free diet, during an even much longer time than the observation period in the above control experiments.

The decrease of the elimination in the stools on yeast-free diet can be explained in two ways. Firstly, the decrease can be assumed to be due to smaller intake of thiamine with the food (only those small quantities which are contained in the autoclaved potato flour). Secondly, it should be remembered that certain factors of the vitamin B complex present in yeast — and to some extent also in autoclaved yeast — are able to stimulate the intestinal synthesis of thiamine (see, for instance, WEGNER *et al.*, 1940).

A comparison of the elimination of thiamine in the stools in the different weight groups reveals distinct quantitative differences. However, if we assume that the elimination in the stools is ascribable — to a major part, at least, on the diets used — to intestinal synthesis, these differences receive a natural explanation, since the extent of the intestinal synthesis is known to be proportional to the size of the coecum (TAYLOR *et al.*, 1942). According to TAYLOR the proportion between the sizes of the coecum in young and old rats may be up to 1 : 50. On this basis, even greater differences could have been expected in the excretion of thiamine in the stools.

With a view to the continuance of the research, the results of these preliminary experiments indicated that rats of the 220 g

(± 10 g) weight group were better suited for elimination studies, since in this group the faecal output of thiamine was higher, and it was therefore easier to detect possible differences in the elimination, caused by the inhibition of the intestinal synthesis by different sulfonamides. On the other hand, the weight group of 110 g (± 10 g) seemed to be more suitable for studies concerning the effect of different sulfonamides on the weight of the animals, since in this group the increase of weight was more marked.

For the above reasons it was decided to use in the following experiments with each preparation rats belonging to both of these weight groups so that both the elimination of thiamine, and the changes in the body weight could be followed.

If was more difficult to choose the diet to be used in the main experiments. However, as the chief object of the research was to study the intestinal synthesis of thiamine, the B₁-deficient diet

TABLE 3, showing the effect of certain sulfonamide drugs and related compounds on the faecal elimination of vitamin B₁, and on the weight of the rats. 4 rats in each group.

Weight groups							
220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)
Daily elimination of vitamin B ₁ in faeces per rat γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat γ	Weight, g
2.9	147	3.3	152	1.9	139	1.5	142
2.1		3.1		2.7		2.8	
Salazopyrine 0.1 g/100 g		Sulfaguanidine 0.1 g/100 g		Sulfathiazole 0.1 g/100 g		Sulfametine 0.1 g/100	
0.6		1.7		2.2		2.3	
0.2		1.2		1.9		2.8	
0	162	1.0	171	1.1	149	1.5	144
0		0.5		1.4		1.1	
0		0.3		0.9		0.9	
0		0.2		0.7		0.7	
+	171	0.3	178	0.4	157	0.9	149
0.5		0.5		0.7		0.9	
0		0.2		0.7		1.0	
0	188	0.4	189	0.6	167	0.8	155

TABLE 3. Page 2.

Weight groups							
220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)
Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g
3.1	152	2.4	146	3.3	140	2.8	144
2.8		2.9		2.1		2.9	
Uliron 0.1 g/100 g		Sulfapyridine 0.1 g/100 g		Neo-uliron 0.1 g/100 g		Sulfadigesine 0.1 g/100 g	
1.9		2.3		2.1		2.3	
2.1		1.9		2.7		2.7	
2.0	150	2.0	158	2.3	159	2.1	159
1.5		1.3		2.1		2.4	
1.1		1.5		2.0		2.3	
1.4	159	1.4	167	1.7	165	1.2	167
1.1		1.1		1.5		1.6	
0.9		0.9		1.8		1.1	
1.3		1.4		1.4		1.3	
1.1	164	1.4	174	1.3	171	1.0	183

TABLE 3. Page 3.

Weight groups							
220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)
Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g
3.3	151	1.9	153	2.2	143	2.9	149
2.9		3.1		3.4		1.8	
Sulfadimine 0.1 g/100 g		Albusid 0.1 g/100 g		Marfanil-prontalbine 0.1 g/100 g		Sulfanilamide 0.1 g/100 g	
2.9		3.1		3.1		1.9	
2.1		2.9		2.7		2.7	
1.9	157	3.3	161	3.3	151	3.4	159
1.9		1.9		2.4		1.8	
2.4		2.1		2.1		1.9	
1.8	165	2.7	169	2.7	159	3.1	166
1.1		2.1		2.8		2.7	
0.9		2.4		3.3		2.1	
1.3		2.9		1.9		1.9	
1.5	174	3.2	181	2.4	164	2.5	170

TABLE 3. Page 4.

Weight groups					
220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)	220 g (± 10 g)	110 g (± 10 g)
Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g	Daily elimination of vitamin B ₁ in faeces per rat, γ	Weight, g
2.7	151	3.1	143	3.1	149
3.5		3.8		3.7	
Marfanil, pur. 0.1 g/100 g		Sulfanilic acid 0.1 g/100 g		p-Aminobenzoic acid 0.1 g/100 g	
2.1		3.2		3.1	
2.7		4.1		4.3	
2.1	159	2.7		5.7	
3.5		3.3	158	3.2	157
2.7		2.5		4.1	
2.1	172	2.2	169	4.5	171
2.1		2.0		4.8	
2.7		3.1		5.1	
2.3		2.8		5.3	
2.8	184	3.1	181	5.1	182

was indicated. On this diet, the elimination of thiamine in the urine fell, in both weight groups, to a definite constant level, at least within the second week from the change of the diet. It was necessary, therefore, to keep the rats on the B₁-deficient diet for two weeks prior to the start of the main experiments.

B. *The main experiments* were planned chiefly on the basis of the experience from the preliminary experiments. Each preparation was tested on 4 rats from both weight groups. When the animals had been two weeks on the B₁-deficient diet, the elimination of thiamine in the urine and stools was estimated twice, at intervals of 2 days, before the preparations were taken into use as inhibitors of the intestinal synthesis. The actual experimental period was 3 weeks. The changes of weight were observed weekly while thiamine was determined every other day. The daily dose of the preparation was 0.1 g per each 100 g of the body weight. The preparation was carefully mixed with the food ration.

Effect of different compounds. Table 3 shows results obtained with different sulfonamides and with sulfanilic and p-aminobenzoic acids. The results of the urine analysis are not included in the tables since no differences could be noted, with any of the

preparations, in the elimination of thiamine in the urine. With rats of the 110 g (± 10 g) weight group, only the effect of the preparations on the body weight was observed. It will be seen from the tables that salazopyrine has lowered most the elimination of thiamine in the stools. Distinct effects were noted also with sulfaguanidine, sulfathiazole, sulfamethine, uliron, sulfapyridine, neo-uliron, sulfadigesine and sulfadimine. On the other hand, no effect was obtained with albucid, marfanil-prontalbine, sulfanilamide, marfanil.pur. and sulfanilic acid. Feeding of *p*-aminobenzoic acid caused a distinct increase of the elimination of thiamine in the stools.

Discussion.

It was mentioned earlier that MacLEAN and BENESCH (1944) were the first to associate the inhibition of the intestinal thiamine synthesis by sulfaguanidine with the central and peripheral neurotoxic complications noted in the patients. NAJJAR and HOLT (1943) showed that human subjects on thiamine-free diet could utilise the thiamine which was synthesized in the intestines, and that sulfonamides stopped this synthesis. This effect was due to the bacteriostatic action of the sulfonamides on the intestinal bacteria, which fact has had practical significance in the use of certain sulfonamide drugs (succinyl sulfathiazole, phthalyl sulfathiazole, sulfaguanidine) in intestinal affections and in the preparation of the large intestine for surgery (e.g. POTH, 1945).

My rat experiments with a number of available sulfonamides and, in addition, sulfanilic and *p*-aminobenzoic acids, showed that the elimination of thiamine in the faeces is stopped most effectively by salazopyrine. This is by no means surprising in view of the encouraging results obtained with salazopyrine in the treatment of ulcerative colitis and certain rheumatic affections (SVARTZ, 1941, 1942, 1, 2), in which its favourable effect is probably due to its action on focal infections in the intestinal tract. CUGNINI (1942), however, maintains that the effect of salazopyrine on the intestinal *coli*-typhoid flora is less than that of sulfapyridine. In spite of the fact that salazopyrine stopped completely the elimination of thiamine in the faeces, no effect was noted in the urinary

elimination. *p*-Aminobenzoic acid increased distinctly the elimination of thiamine in the faeces but had no effect on the urinary elimination.

With preparations having a bacteriostatic effect on the bacterial flora in the intestines, this effect generally became manifest already during the first days of the experiment. Had the faecal elimination once been decreased by sulfonamide, it did not rise again to its normal level during the experimental period. These observations are in agreement with reports on the bacterial flora of the faeces (GANT *et al.*, 1943; MILLER, 1944), according to which the counts of the thiamine-synthesizing *E. coli* decreased from the 3rd day on and remained low for about 3 weeks, to rise again to the earlier level. However, the synthesizing ability of the organism did not return.

A comparison between the inhibition by different preparations of the intestinal synthesis of thiamine — and probably also of most other vitamins of the B complex — and the changes in the body weights of the rats, reveals no correlation (e.g. salazopyrine and sulfanilamide). It is probable that the changes in weight are influenced not only by the inhibition of the intestinal synthesis but also by the toxicity of the preparation used (cf., for instance, *p*-aminobenzoic acid).

If we compare those amounts of thiamine that were eliminated in the urine of the rats on standard diet, with the slight changes in the intestinal synthesis caused by the sulfonamides — with the exception of salazopyrine — it seems difficult to conceive that, at least on a normal diet, the inhibition of the intestinal synthesis could play any decisive rôle in the appearance of those symptoms which accompany sulfonamide therapy and are interpreted as symptoms of B₁-deficiency. This becomes increasingly clear when we observe that if coprophagia is excluded the intestinal synthesis cannot protect the appearance of B₁-deficiency symptoms in the rat, and that the urinary elimination of thiamine is not influenced either by a complete inhibition of the intestinal synthesis (salazopyrine) or its increase (*p*-aminobenzoic acid). It is probable that the major part of the thiamine eliminated in the faeces is present in such a form, for instance in bacteria, as cannot be directly utilised by the organism, at least to any marked extent, although

some absorption might take place in the large intestine (e.g. NAJ-JAR and HOLT, 1943).

S u m m a r y.

1. The following compounds were studied with regard to their effect on the synthesis of thiamine in the intestines of the rat: sulfanilamide, sulfapyridine, sulfathiazole, sulfametine (sulfamethyl thiodiazole), salazopyrine (salicyl azosulfapyridine), sulfaguanidine (sulfanilyl guanidine), sulfadigesine (succinyl sulfathiazole), sulfadimine (sulfapyrimidine), uliron, neo-uliron, albuclid, marfanil-prontalbine (containing 90 % sulfanilamide and 10 % marfanil.pur.), marfanil.purum, sulfanilic acid and *p*-aminobenzoic acid. The rats were divided into weight groups of 220 g (± 10 g) and 110 g (± 10 g). In the preliminary experiments, 15 rats from both weight groups, in groups of 5 rats each, were placed on vitamin B₁ control diet, vitamin B-deficient diet and completely B-complex-free (yeast-free) diet, respectively. The urinary and faecal elimination of thiamine, and the changes in the body weight, were observed in each group during an experimental period of 6 weeks.

2. The experiments showed that on standard diet the urinary elimination in the former weight group varied on either side of 3 γ , and the faecal elimination from 2 to 3 γ per rat per day. A transfer into experimental diets decreased the urinary elimination in all groups, while the faecal elimination decreased only after a transfer on the yeast-free diet. There was increase of body weight on both control and vitamin B₁-deficient diets, while on yeast-free diet the weight decreased. The results with the weight group of 110 g (± 10 g) were similar, the faecal elimination being, however, definitely lower on all diets. There was increase of weight on all diets.

3. The main experiments were planned on the basis of the results of the preliminary experiments. For the investigation of the urinary and faecal elimination of thiamine, 4 rats of the weight group of 220 g (± 10 g) were used for each compound, while the changes in weight were followed with 4 rats of the weight group of 110 g (± 10 g). The compounds were given with the food. the dosage being 0.1 g per 100 g body weight of the rat.

4. The experiments showed that particularly salazopyrine, but also sulfaguanidine, sulfathiazole, sulfametine, uliron, sulfapyridine, neo-uliron, sulfadigesine and sulfadimine, lowered the elimination of thiamine in the faeces but not in the urine. No effect was obtained with albucid, marfanil-prontalbine, sulfanilamide, marfanil.pur. and sulfanilic acid. *p*-Aminobenzoic acid increased the elimination of thiamine in the faeces but not in the urine.

If the quantity of thiamine, produced by the intestinal synthesis, is compared with the amount eliminated in the urine, for instance, on standard diet, the extent of the synthesis will be seen to be so small that even its complete inhibition cannot explain, at least in subjects on a normal diet, those complications which occasionally accompany sulfonamide treatment, and are interpreted as symptoms of vitamin B₁-deficiency.

Chapter 2. The metabolic acetylation of sulfonamides.

Outlining of the problem.

The above experiments on the intestinal synthesis of thiamine showed that — at least with subjects maintained on a normal diet — inhibition of this synthesis by the sulfonamides cannot satisfactorily explain the neurotoxic complications which occasionally result from sulfonamide therapy. It was therefore interesting to study *whether the sulfonamides were possibly associated in some ways with the vitamin B₁ metabolism of the organism.*

Already JUNG (1940) presented a speculation according to which vitamin B₁ would have a central position in the acetylation of sulfonamides. He ascribed the neurotoxic complications of the sulfonamides to the fact that their acetylation would inhibit the conversion of thiamine into its "active form", acetyl thiamine. Although JUNG's theory was based on a wrong hypothesis (cf. STERN and MELNICK, 1940), the author has in the following tried to study the other interrelations between the acetylation of the sulfonamides and the vitamin B₁.

The acetylation problem has a "detoxication" reaction of sulfonamides and as a general metabolic reaction of the organism been recently studied by many authors, especially in the Anglo-

American countries (cf. only LIPMANN's investigations in this connection). For a better understanding of my own work presented later, it is necessary to be acquainted with certain questions in connection with the acetylation in general.

General.

When aromatic amines, which are foreign to the organism, undergo acetylation, in preparation for their removal with the urine as detoxication products, their amino group is linked with the $\text{CH}_3\cdot\text{CO}$ -group. The linkage may, however, take place also in the hydroxyl group as, for instance, when choline is acetylated to form acetyl choline (QUASTEL *et al.*, 1936; STELMAN *et al.*, 1937; MANN *et al.*, 1938, 1939, 1, 2). Acetylation has long since been regarded as an important detoxication reaction but its real significance has become apparent only from the recent work with the sulfonamides.

Earlier phases of the problem of acetylation. BAUMANN and PREUSSE (1879), and JAFFE (1879), isolated from the blood of a dog, which had been given bromobenzene, bromophenyl mercapturic acid, which was later shown to be bromophenyl cysteine. COHN (1893) found *m*-acetyl aminobenzoic acid in the urine of rabbits which had received *m*-nitrobenzaldehyde; when *p*-nitrobenzaldehyde was given there were eliminated in the urine *p*-nitrobenzoic acid and *p*-acetyl amino benzoic acid. In the dog, *m*-nitrobenzoic acid is conjugated with glycocholic acid to form *m*-nitrohippuric acid (CROWDLE and SHERWIN, 1923). KNOOP (1910) established the acetylation in the dog of phenyl- α -amino acid, and NEUBAUER and WARBURG (1910) of phenyl-aminoacetic acid. Acetylation has also been shown to occur in the chicken. MUENZEN, CERECEDO and SHERWIN (1925, 1926) studied the acetylation of *o*-, *m*-, and *p*-aminobenzoic acids in the rabbit, dog and man, and found that the dog does not acetylate these compounds, while acetylation of *m*- and particularly of *p*-aminobenzoic acid occurred in man and dog.

HENSEL (1915) was the first worker to study the "chemical" factors which influence acetylation. He fed to the rabbit, simultaneously with the component to be acetylated (*p*-aminobenzoic acid or *p*-aminobenzaldehyde) also precursors of the acetyl group, i.e. either acetic acid or compounds which increase the formation of acetic acid in the organism. Acetic acid increased acetylation by 60—340 %, pyruvic acid by about 30 %, acetoacetic acid ester by about 20 %; acetaldehyde caused no increase. Similar experiments were made by HARROW *et al.* (1927, 1933, 1, 2, 1934, 1937) who found i.e. that ethyl alcohol increased the acetylation of *p*-aminobenzoic acid by 207 %, ethyl ester of acetic acid by 176 %, sodium acetate by 158 %, β -hydroxybutyric acid by 133 %, pyruvic acid by 126 %, glyceric aldehyde by 118 % and lactic acid by 22 %. It was also found that insulin increased almost twofold the elimination of the acetylated form in the

studying the reflection relationships of the pulse wave opens a new path for diagnostics and an understanding of circulatory disturbances.

The significance of the elasticity and of the speed of the wave is made clear from the following. The aorta is regarded as an elastic tube with the cross-section surface Q and the volume elasticity modulus κ . During the systole the heart pumps in blood with the specific weight σ . The following differential equations apply to the relation between pressure and the speed of the volume flow.

$$\begin{cases} -\frac{\partial p}{\partial x} = \frac{\partial i}{\partial t} \cdot \frac{\sigma}{Q} \\ -\frac{\partial i}{\partial x} = \frac{\partial p}{\partial t} \cdot \frac{Q}{\kappa} \end{cases} \quad (W. Weber, v. Kries)$$

x = the coordinate of length; t = the coordinate of time; p = the pressure at the root of the aorta; i = the speed of the volume flow. The turbulence is neglected. From these equations it can be deduced that an advancing pressure-flow wave moves with the speed

$v = \sqrt{\frac{\kappa}{\sigma}}$ and that p and i become proportional according to the equation:

$$p = i \cdot \frac{\sqrt{\kappa \cdot \sigma}}{Q} = i \cdot \frac{v \cdot \sigma}{Q}$$

If the blood flow from the heart to the aorta during the systole were without any reflection to speak of, the volume of blood pumped out would be:

$$\text{Volume} = V = \int_0^t i \, dt = \frac{Q}{v \cdot \sigma} \int_0^t (p - p_0) \, dt;$$

p_0 = the diastolic pressure.

If the speed v increases -- i. e., if the aorta becomes more rigid, the integral $\int_0^t (p - p_0) \, dt$ must increase if the heart is to pump out the same volume. If the rigidity of the aorta is greater at the same diastolic pressure, then $\int_0^t p \, dt$, the »pressure impulse» of the heart, must also be greater in order to force out the same volume.

With p = the end systolic pressure and t = the systole time, V becomes, under the conditions given above, equal to the stroke volume. This fact opens a new way of determining the stroke volume, if the reflections are studied more closely.

trioxide (KLEIN and HARRIS), and, for instance, damage of the liver parenchyma, have been shown to inhibit acetylation. Acetylation is diminished by a deficiency of vitamin B₁ or riboflavin (MARTIN and RENNEBAUM, 1943), and by hunger (REINWEIN, 1932), as well as by an increase of glucuronic acid derivatives (MARTIN, RENNEBAUM and THOMPSON, 1941).

Characteristic features in the acetylation of the sulfonamides.

The acetylation of the sulfonamides is no "detoxication" in the proper sense of the word, since the conjugation of the acetyl group with sulfonamides increases the toxicity of the drug (MARSHALL *et al.*, 1938) destroying, at the same time, its bacteriostatic effect. The ability of the sulfonamides to undergo acetylation has been known ever since the earliest phases of the sulfonamide therapy, on the basis of the acetylation of corresponding compounds — like *p*-aminobenzoic acid. However, MARSHALL *et al.* (1937) were the first workers to isolate acetyl sulfonamide from the urine of a patient treated with sulfonamide. JAMES (1940, 3) found acetyl sulfapyridine in the urine of a patient who had received sulfapyridine. The toxicity of the acetylated form is mainly due to its low solubility and its tendency to form concrements in the urinary ducts (for review see, for instance, KINNUNEN, 1945). Sufficient distinction has generally not been made between the toxic phenomena associated with the acetylation process itself, and the toxicity of the acetylated form. However, JAMES (1940, 1) already was of the opinion that "sulphanilamide kills by sudden withdrawal of acetate precursors whilst the acetyl compound exerts a specific effect. That this is not due to its lower solubility leading to blockage of the kidney tubules, such as occurs with acetylsulphapyridine, is shown by the histological results".

The acetylation of the sulfonamides follows, on the whole, the general laws of the acetylation of aromatic amines, showing, like the latter process, great variations with different species and also great individual differences within the same species. Besides, there are considerable differences in the acetylation of different sulfonamide preparations. MARSHALL (1939) has shown that acetylation of the sulfonamides occurs in man, monkey, rabbit, guinea-pig, mouse, rat, horse, cow, certain species of fish and in chicken, but not in the dog nor in all species of frog; one species of frog was found to acetylate only sulfathiazole (FAILEY *et al.*, 1943). Clinical observations, numerous animal experiments, and particularly the *in vitro* work of KLEIN and HARRIS with liver, show the extent of variation which may be observed in the acetylation of a given sulfonamide compound even within the same species. Attention has also been paid to the different extent of acetylation of the different sulfonamide preparations *in vivo*. In this respect the sulfonamide drugs commonly employed in the clinical practice may be arranged in a

descending order of acetylation, thus: sulfapyridine (30—45 %), sulfathiazole (20 %), sulfamethyl thiazole (20 %), sulfanilamide (15—20 %) and sulfadiazine (10 %). The figures in brackets indicate the approximate content of the acetylated form in the blood with therapeutic dosage.

In view of the toxic effects of the acetylation process itself, and the low solubility and decreased bacteriostatic potency of the acetylated sulfonamides, the ideal preparation for clinical use would — in this respect — be a sulfonamide whose tendency to acetylation is very slight or nil. From this point of view, sulfadiazine is a very near approach to the ideal. Its acetylation is very low (about 10 % in the blood) and its acetylated form is more soluble than the free drug *e.g.* LAWRENCE, 1946).

Acetylation could also be diminished if it were possible to remove from the organism substances which may act as precursors to the acetyl group, or to blockade the acetylation process itself. As a matter of fact, it has been shown that there is competition between acetylation and the formation of glucuronic acid derivatives, which also explains the lower acetylation values obtained when glucuronic acid is fed (MARTIN, RENNEBAUM and THOMPSON, 1941).

Experimental.

1. The methods used in experiments in vitro.

A. Preliminary experiments on the acetylation of sulfonamides in vitro.

Innumerable clinical observations, together with animal experiments both *in vivo* and *in vitro*, have shown that the acetylation varies considerably in different individuals of the same species, and that significant differences occur even in the same individual under apparently identical conditions and with the same compound. Already HENSEL (1915) — experimenting with aromatic amines — points out the difficulties encountered in setting up prolonged acetylation experiments *in vivo*, especially if several supplementary compounds are to be used. In order to avoid these difficulties, and to be able to study simultaneously the effects of certain supplementary compounds *etc.* on the acetylating power of, for instance, rabbit liver, KLEIN and HARRIS (1938) already carried out their experiments *in vitro*.

In view of the fact that the *in vitro*-experiments require the maintenance of a definite experimental procedure, it was neces-

sary to study the experimental conditions in general, before the main work was begun.

The preliminary experiments were divided as follows:

- a. Effect of an addition of glucose to the Ringer-bicarbonate.
- b. Effect of the sulfonamide concentration.
- c. Effect of the duration of the experiments.
- d. Effect of aerobic and anaerobic conditions.
- e. Acetylation experiments with cell-free extracts of rabbit liver, and with the kidney, spleen and muscle tissue of the rabbit.
- f. Acetylation experiments with the liver, kidney and spleen of different animals. (Some acetylation experiments were made also with the human uterus muscle and myoma tissue).

KLEIN and HARRIS carried out their *in vitro*-experiments with liver slices of 0.2—0.4 mm thickness in Ringer-bicarbonate containing 200 mg glucose per 100 ml. 30—100 mg, dry weight, of tissue were used, and 3 or 4 ml of fluid. The containers, 50 ml erlenmeyer flasks, were shaken constantly in water bath at 37.5°. The pH of the solution was 7.1.

In order to reduce the experimental error, a considerably greater quantity of tissue — 3 g (fresh weight) of fresh liver — was used in the present work. The technical work was facilitated by using, instead of liver slices, a tissue pulp obtained by mincing the tissue with sharp scissors as homogeneously as possible into a mass, the diameter of the pieces being 2—3 mm.

a. *Addition of glucose* has generally been recommended in tissue experiments *in vitro* (KREBS and HENSELEIT, 1932). In order to determine the effect of glucose, the following series of aerobic experiments were made with rabbit liver using sulfa-pyridine (2 mg of the drug per 3 g fresh liver). (Table 4).

Glucose has increased the extent of acetylation, on the average, by 10.3 % (0.8—14.4 %). This finding differs considerably from the results of KLEIN and HARRIS who report that "the process proceeded equally well whether or not glucose was added to the medium. Since *well fed rabbits**) were used, it was probable that the glycogen stores were sufficient to supply the necessary carbohydrate".

*) Italics by the author.

TABLE 4, showing the effect of an addition of 0.2% glucose to the Ringer-bicarbonate on aerobic acetylation experiments *in vitro* with rabbit liver. Time 4 hrs.

Acetylated sulfapyridine, % of total		Percentage increase of acetylation due to glucose
without glucose	with glucose	
37.2	40.2	8.1
43.9	49.7	13.2
41.2	45.9	11.4
59.7	60.2	0.8
39.5	45.1	14.2
26.4	30.2	14.4

b. The effect of the sulfonamide concentration was studied in the following experiments, using 0.5, 1.0, 2.0, 3.0 and 4.0 mg of sulfapyridine per 3 g fresh rabbit liver. The Ringer-bicarbonate contained 0.2 % glucose. Aerobic conditions, time 4 hrs. (Table 5).

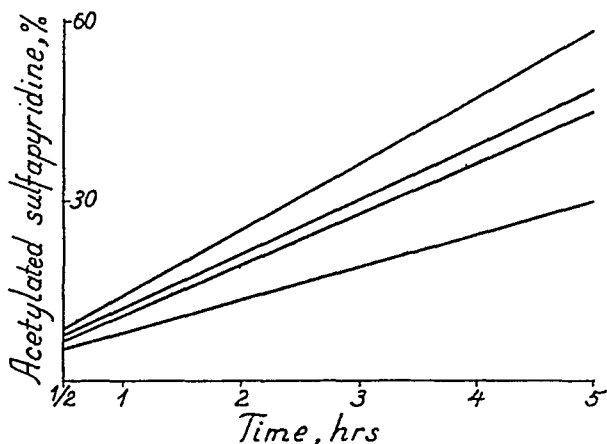
TABLE 5, showing the effect of the sulfapyridine concentration on the acetylation of the drug in aerobic acetylation experiments *in vitro* with rabbit liver.

Added sulfapyridine, mg				
0,5	1,0	2,0	3,0	4,0
Acetylated sulfapyridine, %				
41.7	40.9	40.2	40.0	40.1
50.1	48.1	49.7	47.2	47.0
47.1	45.9	45.9	45.2	44.9
60.2	60.4	60.2	60.1	60.1
46.9	45.8	45.1	45.0	44.3
29.7	30.1	30.9	29.8	29.5

It is evident that with the above concentrations of sulfapyridine — corresponding to 2.5, 5, 10, 15 and 20 mg of the drug per 100 ml of fluid — the acetylation-% is independent of the concentration. Since 10 mg-% probably corresponds most closely to the concentration obtaining in the blood flow in the liver under "physiologic" conditions during therapy, this concentration was employed in later experiments.

c. The effect of time was studied in a number of experiments using the following reaction times: 0.5, 1, 2, 3, 4 and 5 hours. The Ringer-bicarbonate contained 0.2 % glucose. Aerobic conditions: 2 mg sulfapyridine per 3 g fresh rabbit liver. The results are illustrated by graph 1.

GRAPH 1, illustrating the effect of time on the acetylation of sulfapyridine. *In vitro*-experiments with 4 rabbit livers.



With the drug concentration used, acetylation seems to be directly proportional to the time, at least up till 4—5 hours. For practical reasons and in order to avoid autolysis, a reaction time of 4 hrs was not exceeded in later experiments.

d. In order to determine the effects of aerobic and anaerobic conditions a number of experiments was run with the same rabbit livers in atmospheres of oxygen and nitrogen. The oxygen from the oxygen bomb was passed through washing-bottles, while the gas from the nitrogen bomb was kept for at least 24 hrs in a bottle where the last traces of oxygen were removed by yellow phosphorus. The Ringer-bicarbonate contained 0.2 % glucose; 2 mg sulfapyridine were used per 3 g fresh rabbit liver. (Table 6).

Anaerobic conditions lowered the acetylation, on the average, by 29.8 % (21.5—40.6 %) as compared to the aerobic experiments. Subsequent experiments have therefore been made in an atmosphere of oxygen, if not stated otherwise.

e—f. Acetylation experiments were made also with *cell-free* extracts of rabbit liver, and with the kidney, spleen and muscle

TABLE 6, showing the effects of aerobic and anaerobic conditions on the acetylation of sulfapyridine with rabbit liver *in vitro*. Time 4 hrs.

Acetylated sulfapyridine, % of total		Decrease of acetylation in anaerobic experiments, %
aerobically	anaerobically	
49.5	29.4	40.6
52.5	41.2	21.5
38.0	27.5	27.6
20.0	14.3	28.5
58.2	41.2	29.3
61.2	42.0	31.4

of the rabbit. No acetylation could be observed, however. Neither could any acetylation be found to occur in experiments with the liver, spleen and kidney of the horse, cow and pig, nor with rat liver. It may be mentioned that some experiments with human uterus muscle and myoma tissue likewise gave negative results.

Conclusions from the preliminary experiments. On the basis of these preliminary experiments, the following procedure was used in the subsequent series of *in vitro* experiments:

The rabbits were killed by a blow on the head followed by decapitation. The peritoneal cavity was then immediately opened, the liver cut off and the gall bladder, gall duct and the larger veins were removed. The remaining liver tissue was accurately weighed into lots of 3 g each, and cut into a homogeneous mass as described earlier.

The experiment was made with 50 ml erlenmeyer flasks, using a water bath thermostat equipped with a stirring apparatus. Into each flask were pipetted 15 ml Ringer-bicarbonate, prepared according to KREBS and HENSELEIT (1932) and containing 0.2% glucose, and 2 ml of 100 mg-% sulfapyridine, or 2 mg sulfapyridine per 3 g of fresh liver. The liver tissue was quantitatively transferred into the erlenmeyer flask which was then stoppered with a cork through which passed two glass tubes. Oxygen was passed through the fluid during the 4 hours the experiment lasted. The pH of the solution varied from 7.2 to 7.4 (measured with Lyphan) and the temperature of the thermostat from 37.4 to 37.6. After finished experiment the mixture was heated on the boiling waterbath for 3-4 mins to liberate the protein-bound sulfonamide (DRUEY and OESTERHELD, 1942), whereupon it was deproteinised with 20 ml of 20% trichloroacetic acid. The precipitate was filtered or centrifuged off and free and acetylated sulfonamide was determined from the filtrate according to KIMMIG's (1943) thymol method.

KIMMIG's method had, in the preliminary experiments (KINNUNEN, 1946), proved to be a simple, reliable and sensitive procedure which possessed the additional advantage over several others (cf., for instance, FULLER, 1937; MARSHALL, 1937; MARSHALL and BRATTON, 1939; SCHMIDT, 1937; KÜHNAU, 1938; HECHT, 1938; WERNER, 1939; GARDNER and DUNN, 1941), that the simple reagents it required were continuously obtainable in spite of the war.

The control determinations with KIMMIG's method showed that the recovery of sulfonamides added to water accounted to an average of 97–102 per cent, whereas, when added to serum or to liver or muscle pulp, the recovery was — depending upon preparate — only 90–95 per cent. It appears that especially sulfathiazole was inclined to combine with protein being precipitated with it. Neither did the boiling of the proof, as suggested by DRUEY and OESTERHELD (1942), give any better results.

The preliminary tests according to the method of MARSHALL (1937), used in Anglo-American countries, proved that a successful determination depends on the acidity being kept below pH 1 both during the diazotisation, and during the development of the azo dye. The trichloroacetic acid, used as a precipitating agent instead of the *p*-toluenesulfonic acid is, however, decomposed during the hydrolyse to such an extent that pH raises to 2. This increase of the pH-value from 1 to 2 will, again, almost double the extinction value, obtained photometrically. Consequently, the MARSHALL method, otherwise so excellent, could not be applied in these circumstances.

KIMMIG's method is the following:

Necessary reagents: 20 % trichloroacetic acid, 0.5 % NaNO_2 (prepared immediately before use), 0.5 % urea soln., 0.5 % thymol soln. in absolute alcohol, and 40 % NaOH. With the exception of the sodium nitrite, all the solutions are stable.

Into 2 ml of the clear filtrate or centrifugate were added, with occasional shaking, at intervals of 2 mins: 0.2 ml of the nitrite, then 0.2 ml urea to destroy excess nitrite, further 0.2 ml thymol and 0.5 ml NaOH, when a maximum colour immediately developed, suitable for estimation on the photometer. This colour remained unchanged for several hours. The standard consisted of a sample of the filtrate, which was treated in exactly the same way except that distilled water was used instead of the nitrite. Extinction curves were determined separately for each drug, because the colour caused by different preparations differs considerably even when their concentrations are equal. The Pulfrich photometer readings were taken using a 0.5–1.0 cm cuvette and filter S 47.

In order to determine the acetylated form, acid hydrolysis was carried out by adding 2 ml of 2N HCl to 2 ml of the filtrate and heating the mixture for 20 mins on the water bath. After cooling the volume was made up to 4 ml whereupon the estimation was completed as above. In the acid hydrolysis the acetyl group is split off, so that the total amount of sulfonamide present in the solution reacts with nitrite and thymol.

The photometer reading therefore represents total sulfonamide. The acetylated form is obtained from the difference between total and free sulfonamide.

B. Experiments on the acetylation of different sulfonamide preparations with rabbit liver *in vitro*.

General.

In experiments referred to above sulfapyridine was used as a substrate. Especially for practical purposes it would be very interesting to compare the acetylation of different sulfonamides. To obtain a coherent picture of the acetylation of the different sulfonamide preparations, it would be best to make the experiments *in vitro* in order to avoid the difficulties of the *in vivo*-work. It would then be possible, provided that the whole work is done with the liver of the same animal, to create uniform experimental conditions which are the sole basis for reliable conclusions.

Experimental.

Outlining of the problem. The object of the research was to compare the acetylation of certain sulfonamide preparations and closely related compounds (*p*-aminobenzoic acid and sulfanilic acid) in rabbit liver *in vitro*.

Experimental conditions and technique have been described on page 47.

The research was divided into the following parts:

- a. *Preliminary comparative experiments on the acetylation of sulfathiazole and p-aminobenzoic acid.*
- b. *Main experiment with the sulfonamides and chemically related compounds.*
 - a. *Preliminary experiments.* 1. In view of the possibility that the concentration of the different sulfonamides might influence acetylation, a few series of experiments were first set up using different concentrations of sulfathiazole and *p*-aminobenzoic acid. The results are compiled in Table 7.

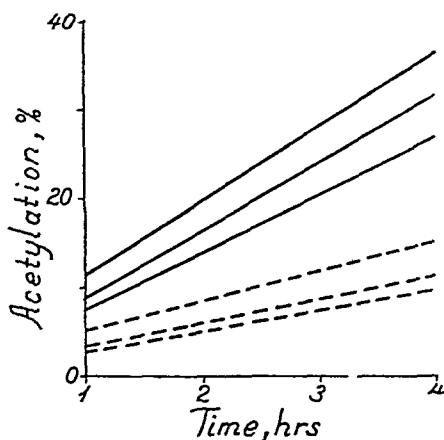
It is seen from the table that in the concentration employed the acetylation of sulfathiazole and *p*-aminobenzoic acid — which were chosen at random — was independent of the concentration.

TABLE 7. Comparative acetylation experiments with 3 rabbit livers and different quantities of sulfathiazole and *p*-aminobenzoic acid. Time 4 hrs.

Compound	mg compound per 3 g tissue			
	1	2	3	4
	acetylated form, % of total			
Sulfathiazole	38.1	37.8	38.1	38.3
<i>p</i> -Aminobenzoic acid	15.2	15.7	15.9	15.0
Sulfathiazole	28.4	28.2	28.9	28.1
<i>p</i> -Aminobenzoic acid	10.5	10.1	10.9	10.1
Sulfathiazole	41.2	41.2	41.7	40.5
<i>p</i> -Aminobenzoic acid	16.4	16.9	16.4	16.1

2. As it could also be expected that *the time of acetylation* might play a rôle (cf. ZEHENDER, 1944) *), preliminary experiments were also made with sulfathiazole and *p*-aminobenzoic acid using acetylation times of 1, 2, 3 and 4 hours. The results of these experiments are illustrated by graph 2.

GRAPH 2, illustrating the effect of time on the acetylation of sulfathiazole (—) and *p*-aminobenzoic acid (----). *In vitro*-experiments with 3 rabbit livers. 2 mg of the compound per 3 g fresh tissue.



*) "Die untersuchten Stoffe unterschieden sich im Tierversuch nicht nur hinsichtlich der Menge an erhaltenem Acetylderivaten, sondern auch hinsichtlich erreichten Endproduktes. Für *p*-Aminobenzoessäure ist letzterer dadurch charakterisiert, dass *schliesslich die Verbindung vollständig in gekuppelter Form ausgeschieden wird*. Für Sulfathiazol und Sulfanilsäure wird dagegen dieser Grenzwert nicht erreicht."

TABLE 8. Acetylation of some sulfonamides and of sulfanilic and *p*-aminobenzoic acids by rabbit liver tissue *in vitro*, in the order of descending acetylation. 2 mg of the drug per 3 g fresh liver.

Compound	No. of rabbit										Average acetylation, %
	26	31	32	33	34	35	36	38	39	40	
	Acetylated sulfonamide, % of total										
Sulfapyridine ...	18.2	40.1	37.7	32.0	24.1	57.2	40.1	47.2	44.3	39.8	38.1
Na-sulfapyridine	18.9	41.0	38.1	31.7	23.1	56.4	42.2	47.2	45.3	38.9	38.3
Sulfaguanidine ...	15.7	36.9	31.4	27.1	19.5	45.0	40.0	42.7	36.4	31.8	32.7
Sulfathiazole.....	17.2	28.7	25.1	20.3	16.0	37.1	32.1	31.8	29.5	23.0	26.1
Na-sulfathiazole	17.1	26.9	24.8	20.0	16.5	37.2	32.1	31.0	29.9	23.7	26.0
Sulfanilamide ...	12.1	20.0	18.5	17.3	12.5	32.1	27.4	27.1	23.7	20.2	21.1
Na-sulfanilamide	11.7	18.9	18.1	17.4	13.1	30.9	26.7	28.0	23.7	21.1	21.0
Albusid	10.3	16.9	16.4	15.1	10.0	25.7	25.0	21.9	19.8	18.5	18.0
Uliron	7.4	12.4	13.5	12.1	7.8	21.3	23.0	17.7	15.4	17.1	14.8
Neo-uliron.....	6.9	10.4	11.2	9.4	7.0	19.0	18.7	15.9	14.1	16.3	12.9
<i>p</i> -Aminobenzoic acid	6.2	7.4	9.5	7.1	5.0	14.2	15.1	13.2	11.8	11.5	10.1
Sulfanilic acid ...	6.1	7.0	8.4	6.9	4.5	12.1	13.8	13.1	10.4	11.0	9.3
Sulfametidine	6.0	7.1	8.4	6.5	4.8	12.1	13.4	12.0	10.5	10.0	9.1

Graph 2 shows that, within the acetylation times employed, the acetylation of *p*-aminobenzoic acid is directly proportional to time, exactly as is the case with sulfapyridine and sulfathiazole.

b. The main experiments on the acetylation of various sulfonamides were carried out *in vitro* using 10 rabbit livers.

The preparations employed are listed in Table 8 in descending order of percentage acetylation.

2. Investigations on the rôle and effect of vitamin B₁ on the metabolism of the sulfonamides, particularly their acetylation.

Experimental.

The work was divided into the following parts:

- Acetylation experiments *in vitro* with rabbit liver at different seasons of the year.
- Effect on acetylation of crystalline vitamin B₁, added *in vitro*.

c. Effect on the acetylation *in vitro* of vitamin B₁, given to the rabbit *in vivo*.

d. Effect of added vitamin B₁ on acetylation *in vivo*.

e. Effect of vitamin B₁ on the distribution of sulfapyridine in the organism.

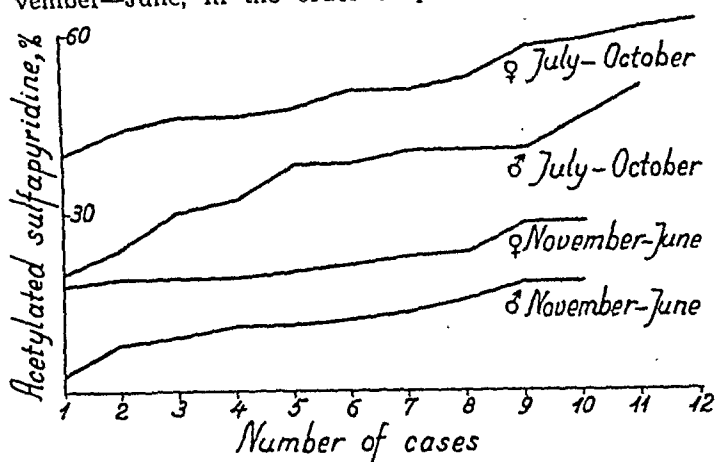
f. Studies on the effect of riboflavin injections on the acetylation *in vivo*.

a. Acetylation experiments *in vitro* with rabbit liver at different seasons of the year. The *in vitro*-experiments were made with livers of rabbits weighing between 1750 g and 2500 g. The rabbits were of the strain of the Department of Medical Chemistry, University of Helsinki. Owing to the exceptional times, the rab-

TABLE 9. Acetylation of sulfapyridine by rabbit liver *in vitro*. The results are given separately for July—October and November—June.

July—October				November—June			
No. of rabbit	Sex	Weight, g	Acetylated sulfapyridine, %	No. of rabbit	Sex	Weight, g	Acetylated sulfapyridine, %
9	♂	1750	40.2	25	♀	2150	19.2
10	♀	1900	49.7	26	♀	2300	18.2
12	♀	1750	45.9	28	♂	1900	10.9
13	♀	2100	60.2	29	♂	1850	10.8
14	♂	2300	45.1	30	♂	1950	8.7
16	♂	2150	30.2	41	♀	2100	21.2
17	♀	1800	49.5	42	♀	2150	22.4
18	♀	1850	52.5	43	♀	2500	28.3
19	♂	1900	38.0	44	♂	2350	3.0
20	♂	2100	20.0	45	♂	2100	17.9
21	♀	2150	58.2	46	♂	2350	18.1
22	♂	1800	50.2	47	♀	1800	19.4
23	♀	1850	45.9	48	♀	1750	20.2
24	♀	1850	61.2	49	♂	1900	7.8
31	♀	1950	40.1	50	♀	1850	19.3
32	♂	2100	37.7	57	♂	1950	12.2
33	♂	1750	32.0	59	♂	2050	15.3
34	♂	1900	24.1	84	♀	1850	22.9
35	♀	1950	57.2	94	♀	2250	28.4
36	♂	2200	40.1	95	♂	2300	13.2
38	♀	2450	47.2				
39	♀	2300	44.3				
40	♂	2100	39.8				

GRAPH 3 illustrates the results of acetylation experiments *in vitro*, separately for male and female rabbits and for July—October and November—June, in the order of percentages acetylation.



bits were kept over the winter on a monotonous hay-and-oats feeding with very little roots in between. In summer they were given fresh grass and in autumn, in addition, swedes and turnips. Water was given *ad libitum*.

Table 9 and graph 3 show the results of acetylation experiments *in vitro* with rabbit liver, using sulfapyridine. The results of experiments during July-October are given side by side with those from November-June. The former series was made with 23 livers of rabbits, of which 12 were female and 11 male. In the latter series 20 rabbits were used, 10 female and 10 male.

These experiments on the acetylating power of rabbit liver *in vitro* show that:

The acetylating power of rabbit liver is definitely greater in July-October than in November-June,

the livers of female rabbits possess a definitely higher acetylation power than those of male rabbits; this is seen especially clearly from the acetylating values for July-October,

in the weight group of 1750—2500 g the acetylating power is independent of the size of the animal.

b. *Effect on acetylation of crystalline vitamin B₁ added in vitro.* As there was reason to assume that vitamin B₁ might play a rôle in the acetylation of the sulfonamides — a possibility speculated upon already by JUNG (1940) — a series of similar experiments was arranged in which the Ringer-solution contained

TABLE 10, showing the effect of crystalline vitamin B₁ on the acetylating power of rabbit liver *in vitro*.

No. of rabbit	A c e t y l a t e d s u l f a p y r i d i n e , %			
	without B ₁	2.7 mg B ₁	0.27 mg B ₁	0.027 mg B ₁
41	21.2	21.2	21.2	21.0
44	3.0	3.1	3.2	2.9
45	17.9	17.3	17.5	18.1
46	18.1	17.9	18.3	19.0
48	20.2	20.4	20.2	20.2
50	19.3	18.9	19.2	19.4
57	12.2	12.8	12.5	12.1
59	15.3	15.1	14.9	—

2.7 mg, 0.27 mg or 0.027 mg of crystalline vitamin B₁ (Merck, "f. wiss. Zwecke"). Table 10 shows the results of these acetylation experiments with sulfapyridine.

It is obvious that crystalline vitamin B₁ is not able to increase the acetylating power of rabbit liver in experiments *in vitro*, even when this power is low.

c. *Effect on the acetylation in vitro of vitamin B₁ given to the rabbit in vivo.* Since an addition of thiamine *in vitro* evidently had no effect on the acetylating power of the liver, it was near at hand to study whether the vitamin, when given *in vivo*, would produce an effect on the acetylation *in vitro*. For this purpose eight full-grown rabbits, chosen at random, were given daily subcutaneous injections of 25 mg vitamin B₁ (Medica) on 7 successive days in winter. The *in vitro* experiments were then made after an interval of 2-3 days. The results of these experiments are given in Table 11, together with the results of 8 ordinary acetylation experiments, carried out at about the same time.

It can be concluded that injections of vitamin B₁ caused a very marked increase in the ability of rabbit liver to acetylate sulfapyridine *in vitro*.

Subsequent experiments on the general mechanism of acetylation were therefore mostly made with rabbits which were first given a preliminary treatment with vitamin B₁.

TABLE 11, showing the effect of vitamin B₁ when given *in vitro*, on the ability of rabbit liver to acetylate sulfapyridine *in vitro*.

B ₁ -injected rabbits		Controls	
No	Acetylated sulfapyridine, %	No	Acetylated sulfapyridine, %
58	35.7	47	19.4
60	29.6	48	20.2
65	11.8	49	7.8
66	38.1	57	12.2
80	35.8	59	15.3
83	28.1	84	22.9
86	37.2	94	28.4
87	57.8	95	13.2
Mean 34.2		Mean 17.4	

d. Effect of vitamin B₁ on acetylation *in vivo*. As it was found that vitamin B₁ when given *in vivo*, increased effectively the acetylation *in vitro*, the effect of injections of vitamin B₁ on the *in vivo*-acetylation in the rabbit was also studied.

The experiments were carried out in winter with 16 rabbits, 8 male and 8 female, weighing from 1750 g to 2500 g. 0.5 g of sulfapyridine was given daily to each animal, well mixed with the oats ration. The volume of the urine, and its content of free and total sulfapyridine, were estimated daily. At the same time, observations were made of possible macroscopic hematuria. As it appeared that at the beginning of the experiment it took a couple of days before the rabbits got used to eating their oats-sulfapyridine ration, the animals were first allowed to adapt themselves to the new diet during 3 days, before determinations were made of the urine output and the proportions of the various forms of the sulfonamide in the urine.

The food mixture was given to the animals from high cups in order to prevent the oats — and sulfapyridine with them — to drop to the floor of the cage, in which case part of the sulfapyridine might have been washed into the collecting funnel together with the urine. Water was given freely and its intake was not measured. The water cup was likewise placed fairly high up in order to prevent a splashing-over to the floor of the cage and also prevent the animals from discharging their urine into the water-cup.

Starting from the 5th day of the actual experiment, daily injections of 25 mg vitamin B₁ (Medica) were made subcutaneously during a period of 9—15 days. The results are illustrated by Table 12. Table 13 illustrates the results of control experiments without vitamin B₁ injections.

TABLE 12. Effect of vitamin B₁ injections on the *in vivo*-acetylating power of the rabbit.

[illegible]

TABLE 12. Page 2.

[illegible]

TABLE 12. Page 3.

		Day of expt.																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
							(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
Rabbit No. 70	Sex ♀ Weight 2300 g	41.5	43.8	49.5	39.7	36.2	43.9	49.2	59.4	60.7	65.2	67.5	71.4	62.0	69.5	67.3	71.4	62.1	65.3	64.1	
	Acetylation, %																				
	Total output of sulfa- pyridine, mg	450	470	430	410	430	470	410	370	310	290	280	180	240	260	230	150	200	180	120	
	Volume of the urine, ml	95	89	110	90	83	80	93	75	60	42	20	16	23	28	18	21	33	41	19	
	Macroscopic hematuria	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	
Rabbit No. 72	Sex ♀ Weight 2150 g	49.2	51.3	52.7	49.4	54.3	59.2	62.3	67.4	69.9	64.5	69.4	62.2	70.1	76.8	62.3	54.8	68.4	59.8	63.2	
	Acetylation, %																				
	Total output of sulfa- pyridine, mg	470	440	390	410	370	330	310	290	280	210	280	310	210	190	190	310	320	280	240	
	Volume of the urine, ml	88	110	92	105	80	72	61	59	35	37	29	40	30	12	28	36	25	25	14	
	Macroscopic hematuria	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	
Rabbit No. 73	Sex ♀ Weight 2400 g	38.2	41.3	43.4	39.7	39.2	48.7	51.2	59.7	62.2	69.7	64.2	58.5	70.4	64.8	60.2	57.8	62.4	59.8	61.2	66.1
	Acetylation, %																				
	Total output of sulfa- pyridine, mg	440	470	410	420	450	400	390	350	190	250	270	330	190	90	110	130	40	310	210	230
	Volume of the urine, ml	103	88	75	100	80	90	35	70	20	48	16	30	45	37	57	52	43	38	25	29
	Macroscopic hematuria	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+
Rabbit No. 74	Sex ♀ Weight 2300 g	39.7	43.2	41.5	38.7	44.2	51.3	57.3	55.2	63.4	67.2	61.5	69.4	71.2	67.3	68.1	61.3	70.4	67.2	63.5	
	Acetylation, %																				
	Total output of sulfa- pyridine, mg	390	470	450	460	380	410	390	380	210	190	250	270	180	270	220	240	200	290	240	
	Volume of the urine, ml	97	99	110	87	83	95	84	75	40	28	29	14	20	21	32	45	37	31	49	
	Macroscopic hematuria	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+

TABLE 12. Page 4.

		Day of expt.															
		1	2	3	4	5	6 (1)	7 (2)	8 (3)	9 (4)	10 (5)	11 (6)	12 (7)	13 (8)	14 (9)	15 (10)	16 (11)
Rabbit No. 67 Sex ♀ Weight 1850 g	Acetylation, %	54.2	69.3	63.4	56.2	58.8	67.2	54.4	59.3	63.7	69.9	62.5	61.8	59.0	65.1		
	Total output of sulfa- pyridine, mg	380	310	290	370	340	280	320	310	330	290	280	370	350	380		
	Volume of the urine, ml	78	92	93	110	97	101	58	57	83	95	98	75	63	92		
	Macroscopic hematuria	—	+	+	—	+	+	—	—	—	+	+	—	—	—		
Rabbit No. 75 Sex ♀ Weight 1900 g	Acetylation, %	61.7	59.8	57.4	60.1	59.5	61.2	60.7	62.3	54.4	59.7	63.4	62.1	61.0	58.4	64.3	61.9
	Total output of sulfa- pyridine, mg	400	370	390	330	310	290	330	350	380	390	410	370	360	310	300	290
	Volume of the urine, ml	90	85	98	110	70	68	45	30	52	81	20	68	59	40	19	30
	Macroscopic hematuria	—	—	—	—	—	+	+	+	+	+	—	—	—	+	+	+
Rabbit No. 76 Sex ♀ Weight 1900 g	Acetylation, %	61.2	63.4	58.3	59.9	61.7	54.7	57.1	61.3	63.4	62.9	69.1	63.2	61.1	60.2		
	Total output of sulfa- pyridine, mg	410	410	370	360	400	370	390	290	330	370	390	380	300	270		
	Volume of the urine, ml	110	79	88	94	95	98	92	73	59	101	48	97	90	83		
	Macroscopic hematuria	—	+	+	—	—	—	—	—	+	+	+	+	+	+		
Rabbit No. 77 Sex ♀ Weight 2350 g	Acetylation, %	50.3	57.2	53.1	40.2	60.7	58.7	53.9	60.2	67.0	61.4	57.2	51.4	58.1	49.9		
	Total output of sulfa- pyridine, mg	430	410	430	390	330	410	380	370	350	400	450	450	410	400		
	Volume of the urine, ml	84	81	110	78	105	95	90	83	81	75	68	79	110	105		
	Macroscopic hematuria	—	—	—	—	+	+	—	—	+	+	+	—	—	—		

TABLE 13. The *in vivo*-acetylating power of the rabbit without injections of vitamin B₁.

		Day of expt.																			
		1	2	3	4	5	6 (1)	7 (2)	8 (3)	9 (4)	10 (5)	11 (6)	12 (7)	13 (8)	14 (9)	15 (10)	16 (11)	17 (12)	18 (13)	19 (14)	20 (15)
Rabbit No. 53 Sex ♀ Weight 2050 g	Acetylation, %	41.2	38.5	36.1	30.7	39.5	30.0	29.0	30.8	14.3	12.4	19.0	27.5	11.1	10.0	29.4	21.6	17.2	20.7	19.5	21.3
	Total output of sulfa- pyridine, mg	390	410	370	440	410	400	460	500	350	260	390	410	390	410	450	470	390	410	370	310
	Volume of the urine, ml	103	111	91	78	100	80	140	75	90	90	123	77	39	85	31	67	120	98	110	114
	Macroscopic hematuria	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+
Rabbit No. 78 Sex ♀ Weight 1950 g	Acetylation, %	40.8	37.9	39.5	42.1	34.2	29.2	27.9	30.1	26.5	30.7	22.4	21.3	19.3	20.1	22.5	18.4	19.8	21.2	18.1	24.1
	Total output of sulfa- pyridine, mg	410	390	460	380	400	470	510	460	390	450	440	380	400	480	510	390	420	470	430	400
	Volume of the urine, ml	100	95	112	105	91	110	84	79	93	98	81	69	74	110	120	58	94	89	99	107
	Macroscopic hematuria	—	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—	+	+	—	—
Rabbit No. 54 Sex ♀ Weight 1950 g	Acetylation, %	49.2	43.7	51.0	40.3	44.2	39.7	41.2	44.3	38.2	35.1	35.1	33.7	39.3	32.0	41.9	30.4	30.1	44.7	32.9	33.4
	Total output of sulfa- pyridine, mg	380	410	470	390	440	350	450	460	370	390	410	380	330	490	370	390	410	450	440	400
	Volume of the urine, ml	95	110	107	59	118	110	70	89	88	97	103	39	74	92	102	110	81	89	99	102
	Macroscopic hematuria	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—	—	—	—	—
Rabbit No. 79 Sex ♀ Weight 2200 g	Acetylation, %	38.4	40.3	18.7	31.9	38.1	22.5	23.0	24.0	46.0	28.0	12.7	23.2	15.7	20.0	32.0	24.3	25.0	18.6	30.2	20.1
	Total output of sulfa- pyridine, mg	350	420	520	330	430	500	470	510	460	530	470	320	520	490	480	320	380	420	390	440
	Volume of the urine, ml	95	105	142	75	110	124	44	140	180	135	183	102	120	182	94	75	148	97	110	95
	Macroscopic hematuria	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	+	+	—	—	—

An examination of the results shows that:

in most of the rabbits the acetylating power was increased by subcutaneous injections of vitamin B₁ in large doses (25 mg per day),

no increase was obtained by injections of vitamin B₁ in those rabbits, whose acetylating power in the preliminary experiment was found to be notably high,

in those rabbits, whose acetylating power was increased by injections of vitamin B₁, the total elimination of sulfapyridine showed a parallel decrease, and so did also the output of urine, hematuria was common in rabbits with high acetylation values.

In the preliminary experiments, the acetylating power of female rabbits was generally higher than that of the males, so that the increase of acetylation through injections of vitamin B₁ was more pronounced in the male rabbits.

e. Effect of vitamin B₁ on the distribution of sulfapyridine in the rabbit organism. Since those rabbits, in which vitamin B₁ injections caused a pronounced increase of the acetylating power, also showed a decrease of the urine output and the elimination of sulfapyridine, it was interesting to study, whether in these animals the vitamin B₁ injections had possibly caused an accumulation of the drug in some particular tissue or in the organism in general.

In order to settle this point, the tissues of 4 vitamin B₁-injected rabbits and 4 control animals were examined on the 15th day of the experiment, 24 hours after the last sulfapyridine dose.

The estimation of sulfapyridine in the tissues was carried out in the following manner: 2 g of the tissue was finely ground with quartz sand in a mortar, and the tissue pulp was extracted with 8 ml of boiling alcohol and 10 ml of boiling water. The estimation was then completed in the usual way according to KIMMIG. (Table 14).

It appeared that the total content of sulfapyridine in the tissues of the B₁-injected rabbits was approximately the same as in the tissues of the controls, but the kidney, liver and serum of the former animals had a slightly higher content of the acetylated form. The percentage proportion of the acetylated form in the serum, liver and kidney of the injected animals was 34.6 %, 38.5 % and 47.8 %, respectively, while the corresponding values for the control animals were 26.9 %, 29.5 % and 34.2 %.

TABLE 14. Distribution of free and total sulfapyridine in vitamin B₁-injected and normal rabbits. The results are given in mg per 100 g tissue.

	No. of rabbit	Serum		Liver		Kidney		Spleen		Muscle		Lungs	
		free	total	free	total	free	total	free	total	free	total	free	total
Vitamin B ₁ -injected	55	12.4	20.1	18.4	32.4	29.1	54.4	8.1	10.7	10.1	12.9	21.9	26.1
	56	15.1	24.2	23.0	39.1	33.7	61.3	11.7	14.2	12.5	15.7	25.0	30.4
	68	11.9	19.5	25.1	37.3	29.8	63.0	8.9	12.3	11.1	13.7	24.7	28.9
	71	20.0	27.3	26.1	41.9	31.9	59.5	12.3	13.1	13.2	15.4	30.2	37.3
	Mean	14.9	22.8	23.2	37.7	31.1	59.6	10.3	12.6	11.7	14.4	25.5	30.7
Controls	53	14.0	19.4	19.0	29.4	27.9	39.1	8.9	12.1	12.3	14.1	22.4	25.4
	54	21.5	28.7	30.1	40.7	35.4	52.9	15.0	18.2	11.5	12.3	38.9	43.2
	79	16.2	21.1	20.4	28.0	21.3	41.8	7.2	9.4	11.2	11.7	20.1	27.1
	78	12.1	18.4	21.7	31.0	38.7	53.4	6.1	7.1	13.1	15.2	18.1	24.0
	Mean	16.0	21.9	22.8	32.3	30.8	46.8	9.3	11.7	12.0	13.3	24.9	29.9

On the other hand, numerous crystals of sulfapyridine were found in the pelvis of the kidney and ureters of the animals which had received vitamin B₁ injections. On hydrolysis, these crystals appeared to consist mainly of acetyl sulfapyridine.

f. *Studies on the effect of riboflavin injections on the acetylation in vivo.* When the major part of the above experiments on vitamin B₁ was already brought to conclusion, the author became acquainted with the work of MARTIN and RENNEBAUM (1943), who reported that acetyl sulfanilamide formation was decreased in thiamine and riboflavin deficiency. Further experiments were therefore indicated on the possible effect of over-doses of riboflavin on acetylation.

These experiments were carried out *in vivo* during winter with 6 full-grown rabbits, 3 males and 3 females. The animals received — during the 3rd phase of the above-described *in vivo*-procedure with vitamin B₁ — daily subcutaneous injections of 2 mg riboflavin (Orion). The results are shown in Table 15.

It will be seen that, under the conditions of these experiments, over-doses of riboflavin did not increase the acetylating power of full-grown rabbits *in vivo*. Neither had it any effect on the urine output or on the total elimination of sulfapyridine in the urine.

Summary.

Numerous clinical observations and animal experiments show that the sulfonamides may cause neurotoxic complications, especially when used over a prolonged period. On the other hand, some animal experiments show that these neurotoxic affections which are more likely to occur under certain conditions, particularly after bodily strain, may be effectively prevented or at least reduced by over-doses of vitamin B₁. It is apparent, therefore, that vitamin B₁ is correlated somehow with the sulfonamide metabolism in the organism. This induced the author to study the rôle of vitamin B₁ in the sulfonamide metabolism, particularly in their acetylation.

The experiments were carried out partly *in vitro* according to KLEIN and HARRIS (1938) with the difference that, instead of the thin liver slices they used, the rabbit liver in the present work was cut with scissors into a fine pulp, the diameter of the

TABLE 15. Effect of riboflavin injections on the *in vivo*-acetylating power of the rabbit.

		Day of expt.																			
		1	2	3	4	5	6 (1)	7 (2)	8 (3)	9 (4)	10 (5)	11 (6)	12 (7)	13 (8)	14 (9)	15 (10)	16 (11)	17 (12)	18 (13)	19 (14)	20 (15)
Rabbit No. 81 Sex ♀ Weight 2350 gm	Acetylation, %	38.5	35.3	41.2	40.7	37.8	39.5	41.2	35.7	38.3	39.2	35.1	37.1	36.4	33.5	34.2	31.7	32.4	35.1	37.2	31.3
	Total output of sulfa- pyridine, mg	430	410	390	450	470	420	430	370	360	350	410	310	390	270	330	420	410	470	410	440
	Volume of the urine, ml	110	93	85	92	97	80	84	87	75	81	87	75	84	91	110	85	93	97	91	94
	Macroscopic hematuria	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+
Rabbit No. 82 Sex ♀ Weight 2150 gm	Acetylation, %	44.3	47.2	50.1	43.5	44.8	45.1	46.8	49.3	47.2	49.3	40.7	42.4	49.2	41.2	41.3	39.5	38.7	33.5	34.8	37.2
	Total output of sulfa- pyridine, mg	490	430	420	440	390	370	410	420	440	360	390	370	330	350	370	310	430	410	440	360
	Volume of the urine, ml	87	95	93	110	92	89	86	88	43	57	69	72	75	74	115	110	92	97	94	105
	Macroscopic hematuria	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	—	—	—	—	—
Rabbit No. 83 Sex ♂ Weight 1950 gm	Acetylation, %	42.5	39.7	41.2	43.4	37.8	39.3	41.5	38.7	35.3	37.2	38.4	39.5	36.0	36.9	39.4	37.2				
	Total output of sulfa- pyridine, mg	440	410	370	390	470	420	430	380	370	390	340	430	320	380	360	390				
	Volume of the urine, ml	100	93	114	85	89	92	67	81	87	59	63	74	77	81	75	71				
	Macroscopic hematuria	—	—	—	—	—	—	—	—	—	—	+	+	+	—	—	—	—			

TABLE 15. Page 2.

		Day of expt.													
		1	2	3	4	5	6 (1)	7 (2)	8 (3)	9 (4)	10 (5)	11 (6)	12 (7)	13 (8)	14 (9)
Rabbit No. 88 Sex ♀ Weight 2200 g	Acetylation, %	45.3	49.2	47.3	51.1	48.0	47.5	46.8	49.4	39.8	44.3	43.0	41.2	42.7	44.5
	Total output of sulfapyri- dine, mg	390	450	480	410	420	410	370	390	420	470	360	380	350	460
	Volume of the urine, ml ...	113	100	87	94	68	85	112	77	39	67	98	114	95	91
	Macroscopic hematuria ...	—	—	—	—	—	—	—	—	+	+	—	—	—	—
	Acetylation, %	30.7	33.4	32.1	29.5	35.6	38.4	36.1	33.5	37.2	32.0	31.5	30.4	29.4	34.3
Rabbit No. 89 Sex ♂ Weight 2400 g	Total output of sulfapyri- dine, mg	430	410	480	430	470	380	370	410	420	470	380	350	410	440
	Volume of the urine, ml ...	93	87	88	94	110	86	88	97	84	48	110	81	89	86
	Macroscopic hematuria ...	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Acetylation, %	49.5	43.8	41.7	48.3	46.1	44.4	45.3	41.2	39.7	41.4	38.9	39.7	41.2	42.1
	Rabbit No. 90 Sex ♀ Weight 2250 g	Total output of sulfapyri- dine, mg	380	440	460	430	410	420	360	370	310	430	440	480	380
Volume of the urine, ml ...		81	94	111	89	75	78	81	88	97	114	65	68	74	80
Macroscopic hematuria ...		—	—	—	—	—	—	—	—	—	—	+	+	+	+

pieces being 2-3 mm. In another part of the experiments, either a combined *in vivo-in vitro* technique was employed, or the experiments were made exclusively *in vivo*.

Preliminary acetylation experiments showed that:

rabbit liver, cut into a homogeneous mass, could produce acetylation *in vitro*,

in contrast to KLEIN and HARRIS' results, glucose increased acetylation *in vitro*,

with amounts of sulfapyridine, ranging from 0.5 to 4.0 mg per 3 g fresh liver, the extent of acetylation was practically independent of the amount of the drug,

with amounts of sulfapyridine, ranging from 0.5 to 4.0 mg per 3 g fresh liver, the extent of acetylation was directly proportional to time,

aerobic conditions increased the acetylation,

cell-free extracts of rabbit liver, and the kidney, spleen and muscle tissue of the rabbit, the liver, spleen and kidney of horse, cow and sheep, the liver of the rat and human uterus muscle and myoma tissue did not acetylate *in vitro* under the conditions of these experiments.

The main experiments with livers of 43 rabbits showed the extent of acetylation to be independent of the weight of the animal, but the values for female rabbits were generally higher than those for the males. In addition, a certain correlation was found to exist between acetylation and the season, in that the values obtained in July-October were higher than those in November-June. The explanation for these differences between values for late summer-autumn and for winter-spring probably lies in the diet and its content of vitamins.

In order to elucidate the rôle of vitamin B₁ in the acetylation of the sulfonamides, a number of *in vitro*-experiments was set up by adding to the acetylation system a known amount of crystalline vitamin B₁. This, however, had no effect on the acetylation values. The result is in agreement with the observations of KLEIN and HARRIS.

The rôle of vitamin B₁ was further studied with acetylation experiments *in vitro* using the livers of rabbits which, during 7 days before the experiment, had received daily subcutaneous

injections of 25 mg vitamin B₁. A comparison with simultaneous control experiments showed that the livers of the pre-treated rabbits possessed definitely higher acetylation values. It is probable, therefore, that in order to increase acetylation, vitamin B₁ must be transformed into another form.

Additional investigations on the rôle of vitamin B₁ in the acetylation of the sulfonamides were carried out with pure *in vivo*-experiments, using altogether 16 full-grown rabbits, 8 males and 8 females. The experiment was set up in three phases. During the first phase of 3 days the animals were allowed to adapt themselves to the sulfapyridine-containing food. During the following phase of 5 days, the urine output was measured and estimations were made of the total urinary elimination of sulfapyridine and of the elimination of the free and acetylated forms of the drug. Hematuria was also followed. During the third phase of 9-15 days the animals were given daily subcutaneous injections of 25 mg vitamin B₁ and observations were made of the effects of these injections on acetylation, elimination of total sulfapyridine, the urine output and hematuria. The control animals received no vitamin B₁.

The experiments showed that injections of vitamin B₁ increase acetylation in rabbits which, during the second phase, exhibited low acetylation values (about 40 %), but not in rabbits whose acetylation at this stage was high (about 60 %). Since, before the injections, female rabbits generally showed higher acetylation values than the males, the effect of the vitamin was more manifest in male rabbits. It was also found that with increasing acetylation there was a decrease in the output of urine and in the urinary elimination of sulfapyridine. Hematuria increased with increasing acetylation.

Tissue analyses showed that the serum, liver and kidney of rabbits, which had received vitamin B₁ injections, had a slightly higher content of sulfapyridine than the corresponding tissues of the control animals, the increase being mostly in the acetylated form. Crystals of acetyl sulfapyridine were found in the pelvis of the kidney and in the ureters of the animals, which had received injections of vitamin B₁.

The decrease in the total elimination of sulfapyridine and the

urine output, and the increase of hematuria, are probably due to an increased acetylation caused by vitamin B₁ with the resulting concrements in the urinary duct. No general accumulation of sulfapyridine in the organism could be noted.

In view of the observations of MARTIN and RENNEBAUM (1943), a number of experiments were made on the ability of over-doses of riboflavin to increase acetylation in full-grown rabbits *in vivo*. These experiments, carried out during winter, showed that at least under the conditions then prevailing, injections of riboflavin did not increase the acetylation.

Vitamin B₁, whose central position in the metabolism of the animal organism — as an essential condition for the dehydrogenation of pyruvic acid — has been recognised earlier, has thus been shown to act also as a catalyst in the acetylation of the sulfonamides. As the acetylation of the sulfonamides therefore seemed to be associated with the fundamental reactions of the animal organism, further research was indicated on some other factors which influence this acetylation.

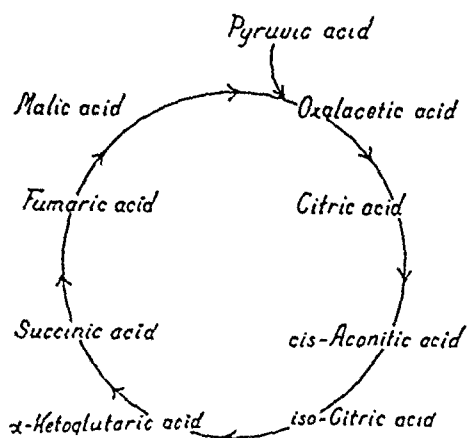
3. *Acetylation of sulfonamides in its relation to the general metabolism.*

The rôle of acetylation in the general metabolism.

Investigations on the origin of the acetylating component and my own work on the rôle of vitamin B₁ in acetylation, all tend to show that acetylation is closely associated with the central reactions of the animal organism. The most important of these reactions — both as regards the metabolism of carbohydrates and, according to latest research, also that of fats — is the tricarmonic acid cycle of KREBS.

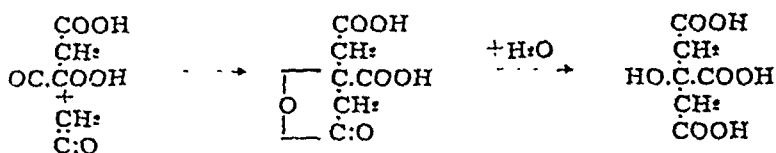
As is well known, the KREBS' cycle is based on the condensation of pyruvic and oxalacetic acids to citric acid, as proposed by KNOOP and MARTIUS (1936). On this theoretical basis KREBS, SALVIN and JOHNSON (1938) built up the following modified scheme for the oxidation of the products of carbohydrate metabolism.

MARTIUS (1943) has recently modified to some extent his views on the mechanism of citric acid formation. According to his latest publications citric acid is not formed through a condensation of pyruvic and oxalacetic acids but through a dehydrogenation of pyruvic acid, followed by a



The tricarboxylic acid cycle of KREBS.

decarboxylation into the ketene radical $\text{CH}_2=\text{C}=\text{O}$. (According to MARTIUS, dehydrogenation should be regarded, in every case, as the first phase of the dissimilation of pyruvic acid in the animal organism). Fusion of this ketene radical with oxalacetic acid produces citric acid lactone which, with water, gives citric acid:



It is interesting to note that MARTIUS here also refers to the possibility that this ketene radical may be an active component both in the formation of acetyl methyl carbinol and in the *acetylation reactions*.

Even in his newest modification MARTIUS thus assumes citric acid to be the first member of the tricarboxylic acid cycle. Recent isotope work has shown, however, that citric acid cannot be an *intermediate* in the tricarboxylic acid cycle. Thus, EVANS and SLOTIN (1940, 1941) and WOOD *et al.* (1941, 1942) showed that the different modifications of the "physiological" oxalacetic acid, $\text{HOOC.CO.CH}_2^{14}\text{COOH}$ and $\text{HOOC.CO.CH}_2^{13}\text{COOH}$, give rise to a Δ -ketoglutaric acid where the isotopic carbon is confined entirely to the carboxyl proximal to the keto group. This result can be explained only by assuming that the primary "condensation" product of oxalacetic and pyruvic acids has an unsymmetrical molecular structure because, if the molecule were symmetrical — as in citric acid — the isotopic carbon in the Δ -ketoglutaric acid would be equally distributed in the two carboxyl groups. Consequently, the first member of the tricarboxylic acid cycle is now assumed to be *cis*-aconitic acid, which may further convert into citric acid or directly into

iso-citric acid. Similar conclusions were drawn, for instance, by LARDY and ELVEHJEM (1945).

The central position of diphosphothiamine in the dehydrogenation of pyruvic acid was established already by LIPMANN (1937), although he erroneously assumed its function to be based on its reversible reduction-oxidation reaction. STERN and MELNICK (1939, cf. also KARRER *et al.*, 1945) showed, however, that dihydro-diphosphothiamine is physiologically inactive. ZIMA *et al.* (1940, 1941) have later suggested that the disulphide form of diphosphothiamine, produced on *oxidation*, would function as an active component in the diphosphothiamine redox-system. By oxidizing diphosphothiamine with iodine they produced this disulphide form chemically and showed that it possessed full vitamin B₁ potency in animal tests. MYRBÄCK *et al.* (1944, 1, 2, 1945, 1, 2) proved that diphosphothiamine exists — in yeast — both in the cocarboxylase form and as disulphide, and the problem thus seems to be settled, since the occurrence of diphosphothiamine in the disulphide form *in vivo* shows that enzymatic oxidation of diphosphothiamine is really possible.

Dehydrogenation ($-2H$) of pyruvic acid — decarboxylation evidently occurs spontaneously — thus leads, in the first place, to MARTIUS' ketene radical *).

The radical theory thus leads from carbohydrates to the tricarmonic acid cycle. However, SIMOLA, has shown years ago (1938, 1, 2) that both butyric acid and β -oxybutyric acid (β -keto acids) are also metabolized via intermediates of the tricarmonic acid cycle. WIELAND and ROSENTHAL (1943), MARTIUS (1943) and BREUSCH (1944) have later arrived at the same result, which was then confirmed by the isotope work of BUCHANAN *et al.* (1945, 1, 2). In fact, MARTIUS assumes citric acid to be formed by two different enzyme systems from pyruvic acid and from β -keto acids.

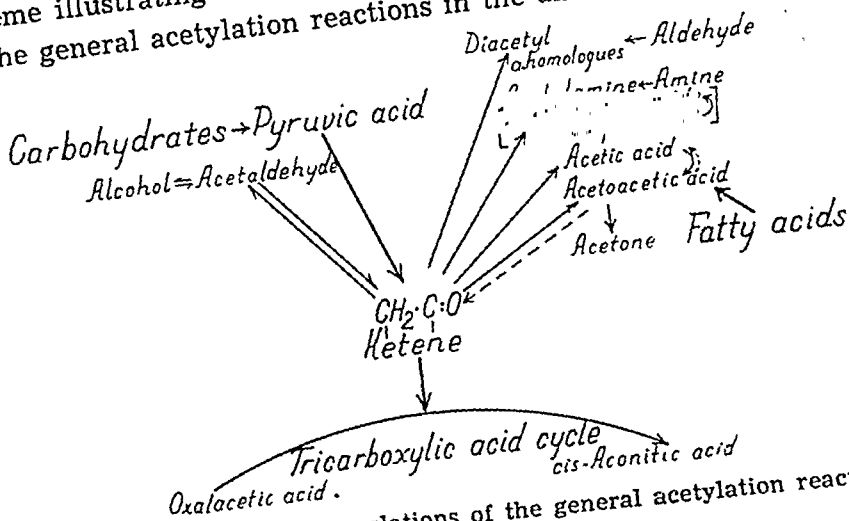
Recent isotope work has also shown that LOEB's (1912) hypothesis of the condensation of acetic acid into acetoacetic acid is correct. The path of acetic acid in the tricarmonic acid cycle therefore evidently goes over acetoacetic acid, which is probably also the way acetic acid functions as an acetylating component. The experiments of BERNHARD (1940, 1) and BLOCH and RITTENBERG (1944), to which reference was made earlier, show that acetic acid really acetylates, although contrary opinions have been expressed by MARTIN and RENNEBAUM (1943) and by DOISY and WESTERFELD (1943).

BERNHARD's (1940, 2) isotope work with deuterio alcohol shows that also ethyl alcohol may act as the acetylating component. For this reason he assumes that ethyl alcohol is dissimilated in the organism over acetic acid. However, in another paper (1942) he reports that ethyl alcohol ace-

*) The relation of this ketene radical, and of the radical $CH_3CO\cdot X$ of LARDY and ELVEHJEM, to LIPMANN's acetylphosphate $CH_3CO\cdot PO_3H_2$, is still unsettled. In this discussion, however, only the general mode of action of the radical need be considered, and not the way it is designated.

lylates more than acetic acid. This obvious discrepancy can be explained by assuming that dehydrogenation of ethyl alcohol would not lead to acetic acid but that acetaldehyde is further dehydrogenated into MAR-TIUS' ketene radical. It is true that this radical could add water and be converted into acetic acid but it could also be transferred directly into the general metabolic reactions, such as the tricarboxylic acid cycle and acetylation.

On the basis of these facts the author proposes the following scheme illustrating the acetylation of sulfonamides as a branch of the general acetylation reactions in the animal organism:



Scheme. illustrating the interrelations of the general acetylation reactions in the organism.

Experimental.

Outlining of the problem. The object of the following experiments was to elucidate — on the basis of the above hypothesis — the acetylation of sulfonamides as a branch of the intermediary acetylations.

The experiments were divided as follows:

- Effect of certain added compounds on the acetylation of sulfonamides *in vitro*.
- Effect of aldehydes on the acetylation of sulfonamides.
- Effect of aerobic and anaerobic conditions on the acetylating power of certain compounds which increase the acetylation of sulfonamides.
- Acetylation of sulfonamides and formation of citric acid as competitive reactions.
- Effect of sulfonamides on the blood sugar level.

a. *Effect of certain added compounds on the acetylation of sulfonamides in vitro.* Ever since HENSEL's (1915) time, attempts have been made to ascertain the effect on the acetylation process of compounds, which may function as possible precursors of the acetyl group. As to the sulfonamides KLEIN and HARRIS studied this question with their *in vitro*-experiments. In the present research the author investigated, from the viewpoint of the above scheme, the effect of certain central metabolites, like acetate, pyruvate, acetyl methyl carbinol, ethyl alcohol and lactate, on the acetylation of sulfonamides *in vitro*.

These *in vitro*-experiments were made with the technique described earlier on p. 47. 2 mg sulfapyridine was used per 3 g fresh rabbit liver. Before a systematic research was begun on the effect of the acetyl precursors on the acetylation of sulfapyridine, it was necessary, however, to decide upon the quantities of the compounds to be used and the duration of the experiment. For this purpose a preliminary experiment was set up with increasing doses of Na-acetate and Na-pyruvate. The results are given in Table 16.

TABLE 16. Effect of different quantities of Na-acetate and Na-pyruvate on the acetylation of sulfapyridine in aerobic *in vitro*-experiments with livers of 2 rabbits.

Added compound	Molar ratio added compound: sulfapyridine				Without addition
	2½	5	7½	10	
	Acetylated sulfapyridine, % of total				
Na-acetate	27.5	29.0	31.2	31.2	24.1
	55.0	57.3	59.5	60.9	46.4
Na-pyruvate	25.1	26.3	27.2	27.4	24.1
	49.7	51.0	52.9	52.7	46.4

There is an increase of the percentage acetylation when the molar ratio *added compound: sulfapyridine* rises from 2½ to 7½, while it remains practically constant when the ratio is from 7½ to 10. In subsequent experiments a ratio of 10:1 was employed.

In order to study the effect of different *acetylation times* a series of experiments was set up using Na-acetate and Na-pyruvate and acetylation times of 1, 2, 3 and 4 hours. Table 17 shows

TABLE 17. Effect of time on the ability of Na-acetate and Na-pyruvate to increase the acetylation of sulfapyridine. *In vitro*-experiments with livers of 3 rabbits.

Added compound	Time, hrs			
	1	2	3	4
	Acetylated sulfapyridine, % of total			
Ringer-soln.	15.1	25.0	36.4	47.5
Na-acetate	15.7	26.7	41.5	58.2
Na-pyruvate	15.4	25.9	38.6	54.1
Ringer-soln.	5.8	8.8	15.7	21.1
Na-acetate	5.8	17.8	24.1	30.4
Na-pyruvate	5.4	11.8	19.1	27.9
Ringer-soln.	11.1	15.6	21.0	23.0
Na-acetate	12.0	17.8	22.4	26.7
Na-pyruvate	12.2	16.3	21.0	23.8

that the increase of acetylation caused by acetate and pyruvate becomes clearly manifest first when the time of acetylation exceeds 2 hrs.

Acetylation was also studied with Na-acetate and Na-pyruvate with and without an addition of glucose to the Ringer-bicarbonate. It was found that the absence of glucose had no effect on acetylation with pyruvate (Table 18).

TABLE 18. Effect of incorporation of glucose in the Ringer-solution on the ability of Na-acetate and Na-pyruvate to increase the acetylation of sulfapyridine. *In vitro*-experiments with livers of 2 rabbits.

Added compound	With glucose	Without glucose
	Acetylated sulfapyridine, % of total	
Ringer-soln.	9.2	7.9
Na-acetate	36.0	30.2
Na-pyruvate	30.7	30.1
Ringer-soln.	43.5	36.4
Na-acetate	66.7	61.3
Na-pyruvate	62.5	62.0

TABLE 20. Effect of acetaldehyde, propionic aldehyde and butyric aldehyde on the acetylation of sulfapyridine in aerobic *in vitro*-experiments.

Added compound	No. of rabbit				
	97	98	99	100	101
	Acetylated sulfapyridine, % of total				
Ringer-soln.	17.3	21.7	39.5	44.7	30.1
Acetaldehyde	15.2	16.0	33.7	39.2	25.4
Propionic aldehyde	16.1	19.5	38.1	39.9	29.7
Butyric aldehyde	17.5	18.9	37.4	40.1	28.4
Na-acetate	—	—	57.4	59.3	—
Na-acetate + acetaldehyde	—	—	51.4	50.1	—
Na-acetate + propionic aldehyde	—	—	52.9	50.7	—
Na-acetate + butyric aldehyde	—	—	54.3	52.0	—

Table 19 illustrates the results of systematic *in vitro*-experiments concerning the ability of Na-acetate, Na-pyruvate, acetyl methyl carbinol, ethyl alcohol and lactate to increase the acetylation of sulfapyridine. It is seen that acetate has increased most the acetylation of sulfapyridine, then come pyruvate, acetyl methyl carbinol, ethyl alcohol and lactate.

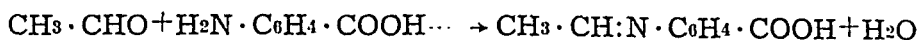
b. It was to be expected that *acetaldehyde* — and other aldehydes as well — could also function as an acceptor to the acetylating component, being converted to diacetyl and further to acetyl methyl carbinol (MARTIUS, 1943; SUOMALAINEN and JÄNNES, 1946, 1, 2). Furthermore, GREEN's *et al.* (1941) work with an enzyme preparation from pig heart has shown that propionic aldehyde is converted to propionyl methyl carbinol (or the isomeric acetyl ethyl carbinol).

In order to study the effect of added aldehyde a number of experiments was made with the usual technique (Table 20). In these experiments, acetyl methyl carbinol was estimated by the method of KNIPHORST and KRUISHEER (1937). The results, however, were altogether negative. *)

Some other explanation must therefore be found for the inhibiting effect of aldehyde on acetylation. Test-tube experiments showed, in fact, that at least acetaldehyde reacts immediately,

*) SIMOLA (unpublished) has shown, indeed, the formation of acetyl methyl carbinol from pyruvic acid in the brain and muscle.

already at the moment it is added to the solution, with the sulfonamides present. In the case of acetaldehyde and *p*-aminobenzoic acid, this reaction is known to lead to the formation of ethylidenaminobenzoic acid (SCHIFF, 1881):



With sulfonamides this reaction evidently proceeds according to the general principles of the formation of SCHIFF's bases. It is also quite probable that NH_2 -group of the sulfonamides is "protected" in this way against acetylation.

c. Effect of aerobic and anaerobic conditions on the acetylating power of certain compounds which increase the acetylation of sulfonamides. In the speculative scheme proposed on p. 71 MYR-BÄCK's and ZIMA's redox-system has been regarded as the necessary prerequisite for the formation of the acetylating component from carbohydrate derivatives. It could be expected, however, that this redox-system would be inactivated in anaerobic conditions. In fact, already KLEIN and HARRIS' experiments show that "there is little anaerobic acetylation compared with the acetylation in aerobic controls". My own preliminary experiments also show that acetylation occurs best in aerobic conditions. When in anaerobic experiments different additions were used, like Na-acetate, Na-pyruvate and acetaldehyde, their acetylating capacity was found to differ substantially from their effect on the same compounds in aerobic conditions. This is illustrated by Table 21.

It will be seen from Table 21 that substrates like pyruvate and

TABLE 21. Effect of some added compounds on the aerobic and anaerobic acetylation of sulfapyridine *in vitro*.

Added compound	Aerobic			Anaerobic		
	No. of rabbit					
	96	110	111	96	110	111
	Acetylated sulfapyridine, % of total					
Ringer-soln.	43.5	30.0	37.2	28.2	15.0	17.5
Na-acetate	66.7	49.2	49.5	37.3	16.7	19.5
Na-pyruvate	62.5	37.4	43.2	41.4	21.7	20.1
Acetaldehyde	39.5	25.1	29.5	40.7	19.7	24.3

acetaldehyde, which themselves may function, not only as the source of the acetyl component, but also as hydrogen acceptors, acetylate in anaerobic conditions *relatively* more than, for instance, acetate which cannot be a hydrogen acceptor. Also KLEIN and HARRIS state that "the decrease in acetylation under anaerobic conditions is, therefore, mainly due to the anaerobic inhibition of acetate formation". They likewise found that pyruvate and acetaldehyde cause a relatively high increase of an acetylation anaerobically, although their interpretation of the facts is hardly the right one.

d. Acetylation of sulfonamides and formation of citric acid as competitive reactions. According to the scheme proposed earlier, the acetylation of sulfonamides is closely associated with the general acetylation reactions of the organism. Consequently, it would be expected that addition of compounds, which themselves may function as acceptors of the acetyl component, decreases the acetylation of sulfonamides and inversely: addition of sulfonamides could be expected to decrease the chances for the radical to be coupled with other acceptors, thereby decreasing, for instance, the formation of citric acid.

Experiments concerning the formation of citric acid were made using the same proportions of added substances as in the acetylation experiments. Simultaneously with each sulfapyridine experiment a parallel was run without sulfonamide. Citric acid was estimated from the trichloroacetic acid filtrate according to PUCHER, SHERMAN and VICKERY (1936, cf. also KREBS and EGGLESTON, 1943). In samples containing sulfapyridine, also acetylated and total sulfapyridine were determined. Samples were taken 15, 30 and 60 minutes from the start of the experiment; because the citric acid formed in the reaction is rapidly destroyed (HALLMAN, 1940). The results are given in Table 22.

We may conclude that sulfapyridine has decreased the formation of citric acid in rabbit liver tissue *in vitro*. The decrease was highest in samples containing Na-pyruvate. This was only to be expected since, according to SIMOLA (1938, 1) and SIMOLA *et al.* (1939, 1, 2), pyruvic acid increases effectively the formation of citric acid.

The same problem, though in an inverse form, was dealt with

TABLE 22. Effect of addition of sulfapyridine on the ability of rabbit liver to form citric acid in aerobic experiments *in vitro*.

No. of rabbit	Substrate	Added compound	Citric acid, γ per 1 g (fresh weight)		
			15 mins	30 mins	60 mins
128	Control	—	40	31	31
	Na-pyruvate	—	94	72	45
	Na-pyruvate	Sulfapyridine	62	45	40
129	Control	—	35	31	—
	Na-pyruvate	—	101	61	—
	Na-pyruvate	Sulfapyridine	84	60	—
130	Control	—	43	37	—
	Na-pyruvate	—	97	69	—
	Na-pyruvate	Sulfapyridine	74	52	—
131	Control	—	37	29	—
	Na-pyruvate	—	64	52	—
	Na-pyruvate	Sulfapyridine	56	42	—

in the following experiment concerning the effect on the acetylation of sulfapyridine of substances which function as acceptors to the acetyl group. According to the scheme, oxalacetic acid is assumed to function as acceptor to the acetyl component, forming citric acid and thereby reducing the chances for the acetylation of the sulfonamides. For practical reasons, however, oxalacetic acid was replaced by its precursor, malic acid.

The experiments were carried out as described in Table 23, using Na-acetate and Na-pyruvate as promoters of acetylation.

e. Effect of sulfapyridine on the blood sugar level. Since the acetylation of sulfonamides is so closely associated with the dissimilation of the carbohydrate metabolites, it was near at hand to assume that sulfonamides would also have an effect on the blood sugar level. This effect has been studied earlier as a separate phenomenon. Thus, SUPNIEWSKI and HANO (1936) report that sulfanilamide increases the blood sugar level. According to MARSHALL (1939), this is undoubtedly due to the use of the very acid hydrochloride of sulfanilamide, and is an acid and not sul-

fanilamide effect, since it can be duplicated with an equimolar amount of hydrochloric acid. di MACCO (1940) has found that sulfanilamide given intraperitoneally increases the blood sugar level of the rabbit by 35-40 %. The effect lasted up till 2 hrs and recurred when a fresh injection was made. On the other hand, JAMES (1940, 1) states that sulfanilamide and sulfa-pyridine given per os lower the blood sugar level, and that this fall is the greater the higher is the concentration of the sulfon-amides in the blood. According to JAMES "the fall in glucose can be explained by glucose being an acetate precursor; this hypothesis is strenghtened by the fact that when acetate is given this fall is prevented".

TABLE 23. The inhibitory effect of malic acid on the acetylation of sulfa-pyridine in *in vitro*-experiments with rabbit liver.

No. of rabbit	Substrate	Added compound	Acetylated sulfa-pyridine, % of total		
			Time, hrs.		
			1	2	3
132	Control	—	16.2	18.2	28.1
	Na-acetate	—	18.9	23.7	34.2
	Na-acetate	Na-malate	8.9	18.3	26.1
	Na-pyruvate	—	17.9	21.2	27.5
	Na-pyruvate	Na-malate	11.0	16.2	20.0
133	Control	—	11.0	15.6	25.4
	Na-acetate	—	14.2	23.5	38.7
	Na-acetate	Na-malate	7.8	17.3	28.0
	Na-pyruvate	—	12.7	19.4	33.2
	Na-pyruvate	Na-malate	6.1	14.4	22.8
134	Control	—	23.1	27.5	36.7
	Na-acetate	—	29.0	37.1	51.3
	Na-acetate	Na-malate	21.3	29.1	41.9
	Na-pyruvate	—	27.2	33.1	44.1
	Na-pyruvate	Na-malate	20.7	26.1	36.3
135	Control	—	31.2	39.1	51.3
	Na-acetate	—	40.8	47.0	67.1
	Na-acetate	Na-malate	25.1	33.1	48.4
	Na-pyruvate	—	36.4	44.5	60.5
	Na-pyruvate	Na-malate	23.7	31.3	41.8

JAMES' view seems very probable indeed. It is more reasonable to assume that the dissimilation of glucose increases with increasing demand for the acetyl group. In order to settle this question a series of experiments was made in which rabbits were given 0.5—1.0 g Na-sulfapyridine as intraperitoneal or intravenous injections whereupon estimation of the blood sugar level were made after 15 and 30 mins and 1, 2, 3 and 4 hrs. The blood sugar was determined according to HAGEDORN and JENSEN (1923) on samples drawn from the ear.

The results are given in Tables 24 and 25. Intraperitoneal injections of Na-sulfapyridine raised the blood sugar level and this effect was still discernible after 3 hours from the injection. In-

TABLE 24. Effect of 0.5 g or 1.0 g intraperitoneally injected Na-sulfapyridine on the blood sugar level of a rabbit, weight 2000—2500 g.

No. of rabbit	Dosage of sulfa-pyridine, g	Blood sugar, mg-%							Maximal increase of blood sugar, %
		Before sulfa-pyridine dosage	Time, hrs						
			1/4	1/2	1	2	3	4	
116	0.5	143	158	179	194	184	151	—	35.7
117	0.5	138	164	185	197	170	155	140	42.8
118	1.0	120	149	181	200	210	160	120	75.0
119	1.0	151	181	200	259	230	180	158	71.5

TABLE 25. Effect of 0.5 g or 1.0 g intravenously injected Na-sulfapyridine on the blood sugar level of a rabbit, weight 2000—2500 g.

No. of rabbit	Dosage of sulfa-pyridine, g	Blood sugar, mg-%							Maximal increase of blood sugar, %
		Before sulfa-pyridine dosage	Time, hrs						
			1/4	1/2	1	2	3	4	
120	0.5	125	147	154	150	142	135	125	23.2
121	0.5	132	147	158	156	144	137	135	19.7
122	1.0	129	151	167	151	151	138	120	29.5
123	1.0	146	178	184	171	159	150	150	26.0

travenous injections likewise raised the blood sugar level though distinctly less than intraperitoneal ones. Larger doses had a more distinct effect. Table 26 indicates the effect of Na-sulfapyridine, given with tube per os, on the blood sugar level.

TABLE 26. Effect of 0.5 g or 1.0 g Na-sulfapyridine, given with tubes per os, on blood sugar level of a rabbit, weight 2000—2500 g.

No. of rabbit	Dosage of sulfa-pyridine, g	Blood sugar, mg-%							Maximal increase of blood sugar, %
		Before sulfa-pyridine dosage	Time, hrs						
			1/4	1/2	1	2	3	4	
124	0.5	120	151	170	165	156	148	122	41.7
125	0.5	132	140	142	142	135	130	128	7.6
126	1.0	139	156	164	159	146	149	144	17.9
127	1.0	127	132	159	172	161	154	130	35.4

That the greater rise caused by intraperitoneal injections was due either to the procedure itself or to an alkali effect, became apparent from experiments in which the same rabbits were given injections of 0.5 ml 1/10 N NaOH, when a similar rise in the blood sugar level could be noted. (Table 27).

TABLE 27. Effect of intraperitoneally injected 0.5 ml N/10 NaOH on the blood sugar level of a rabbit, weight 2000—2500 g.

No. of rabbit	N/10 NaOH, ml	Blood sugar, mg-%					Maximal increase of blood sugar, %
		Before NaOH-dosage	Time, hrs				
			1/4	1/2	1	2	
117	0.5	135	141	155	132	129	14.8
119	0.5	139	158	169	151	142	21.6

The rise of the blood sugar level caused by sulfapyridine can probably be explained in the same manner as the inhibitory effect of aldehydes on acetylation, viz. by a formation of a similar SCHIFF's base. The organism possibly endeavours to compensate for the sugar thus conjugated by raising the blood sugar level. In the estimation of blood sugar, also the sugar component of the SCHIFF's base will react.

Discussion.

The metabolism of the sulfonamides is closely associated with the most central processes of the organism, as evidenced already by the various toxic effects of the drugs (for review see, for in-

stance, KINNUNEN, 1945). Even since the earliest phases of the sulfonamide therapy special attention has therefore been paid to the *acetylation of the sulfonamides and to the mechanical damage caused by the acetylation products*, when acetylsulfonamides precipitate to form concrements which damage, or may even completely blockade, the ureters. However, *very few workers* — JAMES (1940, 1) was the first of them — *have distinguished between the acetylation process itself and the toxic effects of the acetylation products*.

It has been known almost equally long that the use of sulfonamides may cause *neurotoxic complications*. These appear in the form of dizziness, headache, insomnia, peripheral neuritis, rarely optic neuritis (BUCY, 1937), facial paresis (BRUUN and HERMANN, 1942), hypoglossus paresis (PUTKONEN, 1938), respiratory paralysis (MÜLLER, 1945) or convulsions and psychoses resembling epilepsy (GARWIN, 1940). Headache, dizziness, prickly feeling in the legs, and insomnia are often the first symptoms of graver complications. A perusal of literature reveals over a hundred cases of serious neuritis caused by uliron, while about 20 cases were caused by sulfamethyl thiazole and a further 20 cases by sulfapyridine, sulfathiazole and other preparations (MÜLLER, 1945). There are no reports of any neurotoxic complications due to salazopyrine.

The prognosis of pareses caused by sulfamethyl thiazole and uliron is however, relatively good (*e.g.* ELDAHL, 1943, 3), although their frequency is high, as illustrated by the sulfamethyl thiazole material of BROWN and HERRELL (1940), with 3% nervous complications. On the other hand, however, the prognosis of those rarer nervous complications, which are due to non-methylated sulfonamides, is less favourable, since the affects are localised more proximally and involve more pain and disturbances of touch (MÜLLER, 1945).

Of the complications referable to the peripheral nervous system the most frequent is neuritis. At least the most severe cases are motoric, while damage of the sensory nerves is rare (MÜLLER, 1945). The first symptoms referable to the peripheral nervous system appear already during the sulfonamide therapy and consist of paresthetic and neuralgic pains in the extremities; in more

severe cases true nerve paralysis develops. The latter disorder often develops only after a latent period of 2-3 weeks, when the therapy is already finished. The first symptoms generally disappear if the sulfonamide treatment is stopped quickly enough; true neuritis, on the other hand, may last for several months or become chronic.

van VALKENBURG and von dem BORNE (1938) were the first authors to assume that *the neurotoxic symptoms are due to a vitamin B₁-deficiency, caused by the sulfonamides*. This assumption seemed to be supported particularly by certain observations showing that bodily strain played an important rôle in the etiology of the neurotoxic complications (clinical material of TIETZE, 1938; DÖLLKEN, 1938; FOG, 1938; animal experiments of HÜLLSTRUNG and KRAUSE, 1938, 1, 2; ENGELHARDT and BIRKENMAYER, 1938; ENGELHARDT and HÜLLSTRUNG, 1939). The importance of vitamin B₁ in combating the neurotoxic symptoms is illustrated, for instance, by the animal experiments of ENGELHARDT and HÜLLSTRUNG (1939) and BÖSZÖRMENYI (1943), which showed that adequate doses of thiamine prevented, or at least distinctly diminished certain neurotoxic complications caused by sulfonamide preparations.

Sulfonamides have been assumed to cause vitamin B₁ deficiency by lowering or completely inhibiting the intestinal synthesis of thiamine (MacLEAN and BENESCH, 1944), which synthesis has been considered significant, for instance, for man especially when the diet contains little or no thiamine (NAJJAR and HOLT, 1943). My own work on the intestinal thiamine synthesis in the rat showed indeed, that some of the drugs tested, particularly salazopyrine, lowered strongly the elimination of thiamine in the faeces. However, certain other preparations, like sulfanilamide, had no effect whatever in this respect, and *p*-aminobenzoic acid even increased this elimination. Besides, *the synthesis of thiamine in the rat intestines was altogether so slight that even its complete inhibition cannot have any decisive effect on the vitamin B₁ balance of the organism — at least when the animal is given normal diet*. This is quite natural when we remember that already FRIDERICIA *et al.* (1927) showed the intestinal thiamine synthesis to be of importance only when the rats had an opportunity

for coprophagia. It is probable that the major part of the thiamine in the faeces is present in such a form — e.g. in bacteria — as to render its utilisation by the organism impossible. The insignificance of the intestinal synthesis in the thiamine balance of the organism is evidenced also by the fact that the increase of the faecal output of vitamin B₁, caused by p-aminobenzoic acid, had no effect on the urinary elimination.

The bacteriostatic effect of a sulfonamide on the intestinal microbe-flora depends not only on its chemical structure but also on its solubility and resorption and finally also on the bacterial flora itself. The pronounced effect of salazopyrine is not unexpected as it has been known earlier that this drug has a high therapeutic value e.g. in ulcerative colitis (SVARTZ, 1941, 1942, 1, 2). Reports in literature of the effect of the different drugs on the intestinal *coli-flora* — the most important synthesizers of thiamine — vary considerably. According to most reports, sulfapyridine, sulfathiazole and sulfaguanidine have proved most potent. As in the present work these three drugs — amongst the compounds tested — likewise proved most effective, my results are in agreement with the above reports. On the other hand, the effect of sulfadigesine was surprising slight.

FLEISCH and de PREUX (1943) showed by animal experiments that, although thiamine injections had a favourable effect on certain symptoms caused by sulfonamides and resembling those of vitamin B₁ deficiency, they nevertheless seemed to have no effect, for instance, on rat bradycardia. They therefore concluded that *the neurotoxic symptoms caused by sulfonamides are ascribable to some other factor and not to pure vitamin B₁ deficiency*. BRUUN and HERMANN (1943) and MÜLLER (1945) were likewise unable to find in their patients symptoms referable to real vitamin B₁ deficiency.

The above observations were not sufficient to settle the question of the nature of the neurotoxic symptoms caused by the sulfonamides. Attempts have also been made to establish some other connections between sulfonamides and the thiamine balance. Thus, JUNG (1940) was the first to associate speculatively *vitamin B₁ with the acetylation of sulfonamides*. According to his hypothesis, sulfonamides would cause a thiamine deficiency in

the organism by preventing the acetylation of vitamin B₁ and thereby also preventing its conversion into an effective form. This hypothesis was based on the erroneous assumption that acetyl thiamine would be the effective form of thiamine in the organism. However, there is no experimental basis for JUNG's hypothesis. Moreover, STERN and MELNICK (1940) showed that the NH₂-group of thiamine is resistant against acetylation. However, when thiamine was acetylated *in vitro* they found that acetylation takes place at the free OH-group i.e. the same group which is phosphorylated in diphosphothiamine. Since phosphorylation into diphosphothiamine must be regarded as a necessary prerequisite for the conversion of free thiamine into an effective form in the organism, JUNG's hypothesis is obviously altogether groundless.

The substantial part of the present work on the rôle of the acetylation of sulfonamides in the vitamin B₁ balance of the organism was made with rabbits, partly *in vivo* and partly in combined *in vivo*—*in vitro* experiments. During the winters of 1944—46 the animals, owing to exceptional circumstances, were kept on a monotonous hay-and-oats diet receiving roots only occasionally. In summer they received also fresh grass and in autumn turnips and other roots. It is probable that the one-sided hay-and-oats diet in winter caused a state of deficiency which then became apparent, for instance, in the lowered acetylation values. The experiments showed beyond doubt that in rabbits thiamine increases the acetylation of sulfonamides *in vivo* up till three-fold values. This effect was most marked in winter when otherwise the acetylation was less than in summer and autumn.

Thiamine thus occupies a central position in the acetylation of sulfonamides, which again seems to be closely associated with the fundamental reactions of the animal organism, such as the tricarboxylic acid cycle.

KREBS' original theory of the citric acid cycle was chiefly based on the assumption of KNOOP and MARTIUS (1936) that pyruvic and oxalacetic acids are condensed to citric acid. — SIMOLA and his coworkers (SIMOLA and ALAPEUSO, 1938; SIMOLA, HALLMAN and ALAPEUSO, 1939; HALLMAN and SIMOLA, 1939; HALLMAN, 1939, 1940; KRUSIUS, 1940) were again the first to

show the enzymatic synthesis of citric acid from pyruvic acid and C₄-dicarboxylic acids. — According to MARTIUS' (1943) recent views, pyruvic acid is, however, not directly involved but is first dehydrogenated and decarboxylated forming an active ketene radical $\text{CH}_2=\text{C}=\text{O}$ which then reacts with oxalacetic acid. MARTIUS assumes that this radical could function also in acetylation reactions. It may be mentioned that, according to latest research (UTTER *et al.*, 1946; HUNTER and LELOIR, 1945), LIPMANN's (1940) acetyl phosphate could not be found to increase the formation of citric acid.

LIPMANN showed already 1937 that diphosphothiamine occupies a central position in the dehydrogenation of pyruvic acid, although he erroneously assumed its function to be due to its reversible redox-reaction. It has been shown later that dihydrodiphosphothiamine is physiologically inactive (STERN and MELNICK, 1940; KARRER *et al.*, 1945). ZIMA *et al.* (1940, 1941), on the other hand, have shown by animal experiments that the disulphide obtained from diphosphothiamine on oxidation possesses full vitamin B₁ effect. More recently, MYRBÄCK *et al.* (1944, 1, 2, 1945, 1, 2) have reached still farther by showing the occurrence in yeast of an enzyme system which oxidises diphosphothiamine into the disulphide form.

It seems evident, therefore, that vitamin B₁ catalyzes the dehydrogenation of pyruvic acid in the organism by maintaining the redox-system. My experiments showed that injections of vitamin B₁ cause an increase in the acetylation of the sulfonamides. This result is in agreement with the above view of the increased activity of the diphosphothiamine redox-system.

Addition of thiamine hydrochloride *in vitro* had no effect on the acetylation of the sulfonamides — the result being a confirmation of the findings of KLEIN and HARRIS. The explanation was probably that no phosphorylation of thiamine could take place under the experimental conditions employed, although *in vivo* it is known to occur very rapidly, already within 20—40 mins (WESTERBRINK and GOUDSMIT, 1938; OCHOA and PETERS, 1938).

In all probability, the function of the diphosphothiamine redox system is possible only in aerobic conditions. My experiments

in vitro showed, as could be expected, that the acetylation of sulfonamides was definitely more extensive in aerobic conditions than anaerobically. The same observation was made regarding the effect of certain acetyl precursors on acetylation. It was then also found that compounds like pyruvic acid and acetaldehyde, which themselves may act as hydrogen acceptors, increased acetylation relatively more in anaerobic conditions than aerobically, compared, for instance, to acetate.

If the above line of reasoning is followed, then also *citric acid formation should be regarded as an "acetylation reaction", analogous to the acetylation of amines, and it would therefore be expected that the acetylation of sulfonamides would decrease the ability of the organism to form citric acid.* That this is indeed the case, can be clearly seen from my experiments *in vitro*, when pyruvic acid was used to increase the yield of citric acid, according to SIMOLA (1938, 1) and SIMOLA *et al.* (1939, 1, 2).

On the other hand, it could be expected that an incorporation into the experimental system of compounds — like oxalacetic acid — from which citric acid is formed in the organism, would decrease the acetylation of the sulfonamides. For the sake of simplicity, oxalacetic acid was replaced by its precursor, malic acid. The results showed that, also under these conditions, acetylation of sulfonamides decreased.

The acetylating component is formed, not only from carbohydrates — over pyruvic acid — but also from fatty acids. Thus, SIMOLA showed already 1938 that β -keto acids (β -oxybutyric acid) increase the formation of citric acid. This finding has been recently confirmed by WIELAND (1943), MARTIUS (1943), BREUSCH (1943) and BUCHANAN *et al.* (1945). According to MARTIUS the formation of citric acid involves two separate enzyme systems one starting from pyruvic acid and the other from acetoacetic acid (see also HUNTER and LELOIR, 1945). Since recent isotope work has shown that acetic acid is condensed to acetoacetic acid in the organism, it would be near at hand to assume that acetic acid in this manner functions as the acetylating component (cf. HUNTER and LELOIR). From ethyl alcohol the acetylating component is probably formed over acetaldehyde.

Also acetaldehyde — and other aldehydes as well — would be

expected to function as acceptors to the acetylating component (MARTIUS, 1943; SUOMALAINEN and JÄNNES, 1946, 1, 2) by being converted to diacetyl and further to acetyl methyl carbinol. Formation of acetyl methyl carbinol could not be established, however, in my experiments *in vitro*, although acetaldehyde, propionic aldehyde and butyric aldehyde decreased the acetylation of the sulfonamides. The explanation probably is the formation of a SCHIFF's base — occurring already *in vitro* — whereby the amino group is bound and prevented from being acetylated *in vitro*.

It appeared from the discussion of vitamin B₁ that carbohydrates occupy a central position as precursors to the acetyl group. Hence it would have been expected — in conformity with JAMES' (1940, 1) view — that the *increased conjugation of the acetyl groups due to sulfonamides would accelerate the consumption of carbohydrates by the organism and that this would have become manifest, for instance, as a fall of the blood sugar level. However, when rabbits were given Na-sulfapyridine intraperitoneally, intravenously or per os, there was a rise of the blood sugar level.* It is probable that also this rise can be explained by a formation of a SCHIFF's base, so that the organism endeavours to compensate for the glucose, which is bound to the sulfonamides, by raising the blood sugar level. When blood sugar is estimated, also this bound glucose becomes involved in the reaction.

It is apparent therefore that sulfonamides, as easily-acetylated substrates, diminish the natural acetylation in the organism and thereby cause disturbances e.g. in the tricarboxylic acid cycle.

In addition to the acetylation reactions listed above, there occur in the organism other acetylations which — though quantitatively less — are of decisive importance in the life processes. Thus, for instance, the formation of acetyl choline has been found to be an acetylation process which is catalyzed by vitamin B₁ (e.g. MANN and QUASTEL, 1940). Certain pictures of illness, like myasthenia gravis, have already earlier been explained by a decreased acetylation of choline (TORDA and WOLFF, 1944). Furthermore, LIPMANN (1946) showed quite recently that in anaerobic conditions the same enzyme may function in the acetylation of both sulfonamides and choline.

The acetylation of sulfonamides has been shown to cause

disturbances in the citric acid formation. Analogously, the sulfonamides can be expected to prevent the formation of acetyl choline. Hence the basal cause of the neurotoxic complications due to sulfonamides would be their acetylation, whereby the physiological acetylation of choline is prevented. The action of thiamine in preventing or healing these neurotoxic symptoms would thus be explained by the increase it causes in the formation of the acetylating radical, with increasing dehydrogenation of pyruvic acid.

It may be mentioned as an interesting feature that the antipyretic effect of certain sulfonamide drugs has been ascribed to their free NH_2 -group, and that this effect is particularly noticeable with easily-acetylated preparations, especially sulfapyridine (MARX, 1940; BEESON and JANEWAY, 1940; MAJOR, 1940; FLIPPIN *et al.*, 1940; NICOLAI, 1941). It should be observed, moreover, that insulin has been found to increase acetylation (HARROW *et al.*, 1937) whilst, according to KREBS and EGGLESTON (1938), it also catalyzes the tricarmonic acid cycle.

If the ability of different sulfonamides to lower the faecal output of thiamine is compared to their acetylation and the neurotoxic complications they cause, there is in general no correlation. Preparations with the most pronounced bacteriostatic effect on the intestinal bacteria are by no means the most frequent causes of nerve complications. Neither have the most strongly acetylating drugs always been found to cause the most neurotoxic complications.

The etiology of the nerve damage must therefore involve some other factors besides the disturbances in acetylation. This is easily understood if we consider that doses of vitamin B_1 have not produced favourable results in *all* cases. The cause of the negative results must in these cases be sought elsewhere. Thus already MARSHALL *et al.* (1937) pointed out that the cause of the neurotoxic complications would be the individual mode of reaction of the patient, the size of the dose and the secreting ability of the kidney. It has been shown recently, however, that the size of the dose is not always decisive (BRUUN and HERMANN, 1942). MÜLLER (1945) again assumes that at least in part of the cases allergy is the cause of the neurotoxic complications. He supports

his assumption, for instance, by the fact that in part of his cases, earlier anamnesis revealed asthma and other allergic indications, and that most patients had eosinophilia. The possibility of allergy seems plausible indeed in those cases where the complications were preceded by longer latent periods. Also other possible explanations have been suggested. NYMAN (1942) assumes that particularly the *methyl group* would increase the toxicity of the sulfonamides (uliron, sulfamethyl thiazole) as well as of certain other drugs (e.g. cocaine, morphine and strychnine). It should be observed, however, that sulfamezathine, in spite of its two methyl groups and pronounced bacteriostatic effect, is the least toxic of all sulfonamides.

All these theories concerning the etiology of the neurotoxic symptoms caused by sulfonamides illustrate the different sides of the problem. It is very probable, in fact, that the etiology is far from uniform. However, *the manifold effects of the sulfonamides on the life processes of the organism are best illustrated by these toxic symptoms which are indicative of the disturbances the acetylation of sulfonamides causes in the general acetylation reactions of the organism — including also the formation of acetylcholine.*

Summary.

I. 1. Experiments concerning the *intestinal synthesis of thiamine* in the rat showed that more synthesis occurs in big rats (weight group 220 g) than in small ones (weight group 110 g). In the former group the faecal output of thiamine varied from 2 to 3 γ and in the latter from 0.8 to 1.3 γ per rat per day. Within the same weight group, the synthesis was practically the same on standard diet, vitamin B₁ control diet and vitamin B₁-deficient diet, but less on a completely yeast-free diet. On the other hand, a change from one diet to another had a distinct effect on the urinary output of thiamine, which in general assumed a constant level only on the second week from the change — and still later when the animals were transferred on yeast-free diet.

2. *A study of the effect of certain available sulfonamide preparations and of sulfanilic and p-aminobenzoic acids on the syn-*

thesis of thiamine in the alimentary tract of the rat showed that particularly salazopyrine, but also sulfaguanidine, sulfathiazole, sulfametine, uliron, sulfapyridine, neo-uliron, sulfadigesine and sulfadimine decreased the intestinal synthesis, while albucid, marfanil-prontalbine, sulfanilamide, marfanilum pur. and sulfanilic acid had no effect. *p*-Aminobenzoic acid increased the faecal output of thiamine. None of these preparations had any effect on the urinary output of thiamine.

II. A. 1. *Experiments concerning the rôle of vitamin B₁ in the metabolism of the sulfonamides were made with rabbits either in vitro, in vivo or as combined in vitro—in vivo experiments.* The experimental *in vitro*-technique of KLEIN and HARRIS was simplified by using, instead of liver slices, a homogeneous liver pulp. Sulfapyridine was used as the substrate to be acetylated since, according to clinical experience and *in vivo*-experiments, its acetylating capacity was known to be highest.

Preliminary experiments showed that glucose increases the acetylation *in vitro*. With quantities of sulfapyridine ranging from 0.5 to 4.0 mg per 3 g fresh liver, the percentage acetylation was independent of the concentration of the drug and directly proportional to time, at least up till 5 hrs. Anaerobic conditions decreased acetylation. Acetylation could not be established with tissues other than rabbit liver.

2. Great variations were noted in the acetylating power of livers from different animals, although in full-grown rabbits the acetylation was independent of the weight of the animal. The acetylation values were generally higher in July-October than in November-June. They were likewise higher in females than in males.

3. Addition of crystalline thiamine hydrochloride *in vitro* had no effect on the extent of acetylation.

4. Administration of vitamin B₁ *in vivo* (as injections) increased the acetylation *in vitro*.

5. Injections of vitamin B₁ increased the acetylating capacity of the rabbit *in vivo*. This was best observed in animals whose acetylating capacity otherwise was low. With increasing acetylation there was a decrease in the amount of urine and in the urinary output of total sulfapyridine, whereas hematuria increased.

6. In spite of the decreased elimination of sulfapyridine, injections of vitamin B₁ did not cause any accumulation of sulfapyridine in the tissues. The values for the acetyl sulfapyridine content of the serum, liver and kidney were only slightly higher in the thiamine-injected rabbits than in the controls, whilst in the former group precipitation of acetyl sulfapyridine in the ureters explained to some extent the decreased elimination.

7. Injections of riboflavin had no effect on the extent of acetylation.

B. 1. Systematic comparison of the acetylation *in vitro* of different sulfonamides showed that acetylation decreased in the following order (the average acetylation values in brackets) sulfapyridine (38 %), Na-sulfapyridine (38 %), sulfaguanidine (33 %), sulfathiazole (26 %), Na-sulfathiazole (26 %), sulfanilamide (21 %), Na-sulfanilamide (21 %), albusid (18 %), uliron (15 %), neo-uliron (13 %), p-aminobenzoic acid (10 %), sulfanilic acid (9 %), sulfametine (9 %).

2. Compounds, which function as precursors to the acetyl component, were found to increase the acetylation of sulfonamides in aerobic conditions in the following order: acetate, pyruvate, acetyl methyl carbinol, ethyl alcohol, lactate.

3. In anaerobic conditions, compounds which simultaneously may function as hydrogen acceptors (pyruvate, acetaldehyde) increased the acetylation relatively more than, for instance, acetate which cannot function as hydrogen acceptor.

4. Sulfapyridine decreased the ability of rabbit liver to form citric acid. This was clearly noted particularly when pyruvate was employed to increase the citric acid formation.

5. Malic acid decreased the acetylation of sulfapyridine.

6. Acetaldehyde—together with propionic and butyric aldehydes—decreased the acetylation of sulfapyridine in aerobic conditions. This decrease was apparently due to the reaction which occurs already *in vitro* between aldehydes and sulfonamides and leads to the formation of a SCHIFF's base.

7. Intraperitoneal injections of Na-sulfapyridine raised the blood sugar level in rabbits. A slight rise was noted also with intravenous injections or peroral application. That the greater rise of the blood sugar level by intraperitoneal injections was ascrib-

able either to the procedure itself or to the alkalinity of the solution, was demonstrated by intraperitoneal injections of corresponding quantities of sodium hydroxide.

Investigations were made to elucidate the effect of the sulfonamides, and of sulfanilic and *p*-aminobenzoic acids, on the synthesis of thiamine in the rat intestines. It was found that some of the drugs decreased the faecal elimination of thiamine, while others had no effect in this respect, and *p*-aminobenzoic acid caused an increase. *It could be proved that the intestinal synthesis of thiamine in the rat is so slight that — at least on a normal diet — even its complete inhibition cannot explain those symptoms which are occasionally caused by the sulfonamide therapy and held to be symptoms of vitamin B₁ deficiency.*

Investigations on the acetylation of sulfonamides in the organism, and its relation to the metabolic acetylation reactions showed that — in conformity with earlier reports — the acetylating power of rabbits *in vivo*, and of rabbit liver *in vitro* was subject to wide individual variations, which were clearly influenced upon by the state of nutrition. *Vitamin B₁ apparently occupies a central position in the acetylation of sulfonamides.* This is probably due to the fact that thiamine causes an increased formation of the acetylating component from the dissimilation products of carbohydrates, in the first place from pyruvic acid. In experiments *in vitro*, acetate proved to be the best precursor to the acetyl group. Recent isotope research has shown that acetate is condensed to acetoacetic acid. On the other hand, β -keto acids have been shown to increase the formation of citric acid.

In vitro-experiments showed that sulfapyridine decreases the formation of citric acid and that malic acid, on the other hand, decreases the acetylation of sulfapyridine. The acetylation of sulfonamides is thus associated with the central acetylation reactions of the organism.

In all probability, the key substance in the general acetylation reactions of the organism is a reactive radical (the ketene of MARTIUS, the acetyl phosphate of LIPMANN). Fusion of the radical with oxalacetic acid leads to the tricarboxylic acid cycle and to amines, acetyl amines, acetaldehyde (or other aldehydes), over diacetyl to acetyl methyl carbinol (or homologues of di-

acetyl), and to choline and acetyl choline. The acetylating component is probably formed from carbohydrates over pyruvic acid, from ethyl alcohol over acetaldehyde and from acetic acid (and butyric acid) over acetoacetic acid.

According to this line of thought, the neurotoxic complications of the sulfonamides would be explained by assuming that the sulfonamides conjugate with the acetylating component whereby the formation of acetyl choline is prevented. The preventive effect of vitamin B₁ on the development of neurotoxic complications would then be due to its activating effect on the oxidation of pyruvate which causes an increase in the available quantity of the acetylating component. This would complement the picture of the acetylation of the sulfonamides as a process closely associated with the central functions of the organism and as a competitive reaction to the physiologic acetylations. At the same time, certain characteristic complications of the sulfonamide therapy could be better understood.

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